

THE EFFECTS OF INFLAMMATORY MEDIATORS ON HUMAN NASAL SECRETIONS

A Thesis submitted by

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For the degree of Doctor of Philosophy

in the University of London

2003

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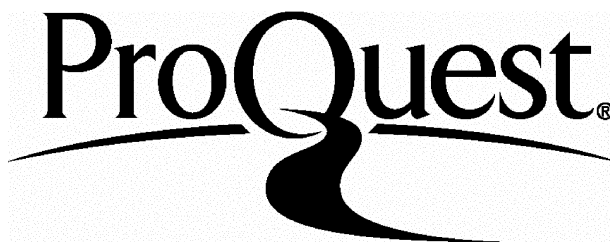
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ABSTRACT

Allergic rhinitis is one of the common allergic diseases. Symptoms of allergic rhinitis are pruritus (itching) of the nose, sneezing, nasal congestion and rhinorrhea. Rhinorrhea is a result of an increase in nasal secretion and is one of the fundamental components of allergic rhinitis. The first aim of this thesis was to quantitatively analyse this parameter of the nasal response following nasal challenge with synthetic inflammatory mediators. In order to quantitatively measure the amount of nasal secretion, a simple and relatively non-traumatic method for collecting the secretion was developed. Insertion of a cotton bud into each nostril proved to be an efficient method for quantitatively analysing an increase in nasal secretion produced following exogenous application of synthetic inflammatory mediators. Secondly, the mechanism by which mediators induced nasal secretion was also examined by analysis of protein content in nasal lavage after mediator administration. An increase in albumin concentration indicated an increase in vascular permeability whereas glandular secretion was evaluated by an increase in lysozyme and lactoferrin (serous cell origin) and fucose for mucous glycoprotein secretion (mucous cell origin). This thesis presents an investigation on the secretory activity of human nasal mucosa and the source of the secretion induced by increasing concentrations of methacholine, histamine and bradykinin in normal human subjects, and substance P in atopic and normal subjects.

An increase in nasal secretion was observed following nasal challenge with methacholine, histamine and bradykinin. Methacholine- and histamine-induced nasal secretion was a mixture of both vascular and glandular proteins while bradykinin-induced nasal secretion contained protein of vascular origin. The antihistamine, cetirizine, and the bradykinin B₂ receptor antagonist, Hoe 140, inhibited histamine- and bradykinin-induced increases in nasal secretion of albumin, respectively. Cetirizine had no effect on histamine-induced glandular secretion. Challenge of one nostril with 1000 µg histamine induced reflex albumin secretion in the opposite, non-challenged nostril. This indicated that histamine may induce neuronal reflexes in normal subjects but a high concentration was needed. The effects of the neuropeptide, substance P, were examined in normal and atopic subjects. Substance P induced a vascular response but not a glandular response. Atopic subjects produced greater responses, albumin release and facial cutaneous blood flow, than in normal subjects.

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ACKNOWLEDGEMENTS

I am so grateful to everyone who has given me his or her support and encouragement but most of all to my supervisor, Professor John Foreman. I would like to thank for his advice, support, patient, encouragement and understanding which he has given me over the years since the final year undergraduate project.

I would also like to thank Mr. Nick Hayes for his untiring technical support and his generous help with my dreadful English.

Thank you to all those who has made this research possible by generously giving their time to be subjects in this study and allow me to use their nose. Without them, this study would have been impossible.

Finally, thank you to my family and my friends for their support and help throughout my time in England and at UCL.

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
Ach	Acetylcholine
AchE	Acetylcholinesterase
AGEPC	Acetyl-glycerol-ether-phosphorylcholine
AUC	Area under the curve
AVA	Arteriovenous anastomoses
BK	Bradykinin
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX	Cyclo-oxygenase
CPN	Carboxypeptidase N
4-DAMP	4-diphenylacetoxy-N-methylpiperidine methiodine
Delta-R	Delta-reductase
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme linked immunosorbent assay
EPO	Eosinophil peroxidase
FLAP	5-lipoxygenase activating protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMWK	High-molecular-weight kininogens
HMW-NCA	High-molecular-weight neutrophil chemotactic activity
5-HPETE	5-hydroperoxyeicosatetraenoic acid
HRP	Horseradish peroxidase
H ₂ SO ₄	Sulphuric acid
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
KD	Kallidin
LMWK	Low-molecular-weight kininogens
LT	Leukotriene
MBP	Major basic protein
MC	Mast cell
MCP	Monocyte chemotactic protein
MGP	Mucous glycoprotein
MIP	Monocyte inflammatory protein
MPO	Myeloperoxidase
NAP-2	Neutrophil-activating peptide-2
NAR	Nasal airway resistance
NEP	Neutral endopeptidase
NKA	Neurokinin A
NKB	Neurokinin B
NO	Nitric oxide
NPY	Neuropeptide Y
O ₂	Oxygen
OPD	<i>o</i> -phenylenediamine dihydrochloride substrate
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PG	Prostaglandin
PGDH	15-hydroxydehydrogenase
PHM	Peptide histidine methionine
PLA ₂	Phospholipase A ₂
PT	0.05% Tween 80 in PBS, pH 7.4
RANTES	Regulated on activation, normal T expressed and secreted
sIgA	Secretory immunoglobulin A
S.E.M.	Standard error of the mean
SP	Substance P

TC	Time course
TNF α	Tumor necrosis factor-alpha
TX	Thromboxane
VIP	Vasoactive intestinal polypeptide

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CHAPTER 1

INTRODUCTION

1.1 ANATOMY AND PHYSIOLOGY OF THE HUMAN NOSE

1.1.1 The anatomy

The nasal septum divides the nasal cavity into two separate compartments: the left and the right cavities. Each nasal cavity can be divided into three parts: the nasal vestibule, the olfactory region and the respiratory region. The nasal vestibule is the transition zone between the external and internal environment. It forms the external nose which projects from the face, and it resembles a pyramid with its base at the facial skeleton and its apex projecting anteriorly. The apex of the nose is formed by two pairs of nasal cartilages. The upper lateral nasal cartilages attach to the nasal bones and articulate with one another in the midline, to provide the shape of the middle third of the external nose. The lateral crura of the lower lateral cartilage maintains the patency of the nasal vestibule. Small muscle groups contribute to the shape and function of the external nose; these muscles all function to dilate the nostrils. The olfactory region and the respiratory region form the internal nasal cavity. The olfactory region has a small surface area compared with that of the respiratory region. The rest of the internal nasal cavity constitutes the respiratory region. The nasal “valve” is a constriction separating the external nose from the internal nasal cavity, and is an important area since it limits the rate of inspiratory nasal airflow and accounts for approximately half of the total resistance to respiratory airflow.

There are three bony structures arising from the lateral nasal wall of the internal nasal cavity. They are referred to as the inferior, middle and superior turbinate bones. Each turbinate is lined with pseudostratified columnar epithelium. In addition to increasing the mucosal surface of the nasal cavity, the turbinates also function to regulate airflow. An increase in turbinate size reduces air flow and vice versa. The inferior

turbinate bone is the most important since it is the largest of the three and is the point at which the nasal cavity is at its most narrow.

1.1.2 The histology

The nasal vestibule is lined by skin from which hair grows. Beneath this skin is the respiratory mucous membrane: the nasal mucosa. The nasal mucosa may be divided into three major segments, the surface epithelium, the lamina propria or the submucosa and the basement membrane, figure 1.1.

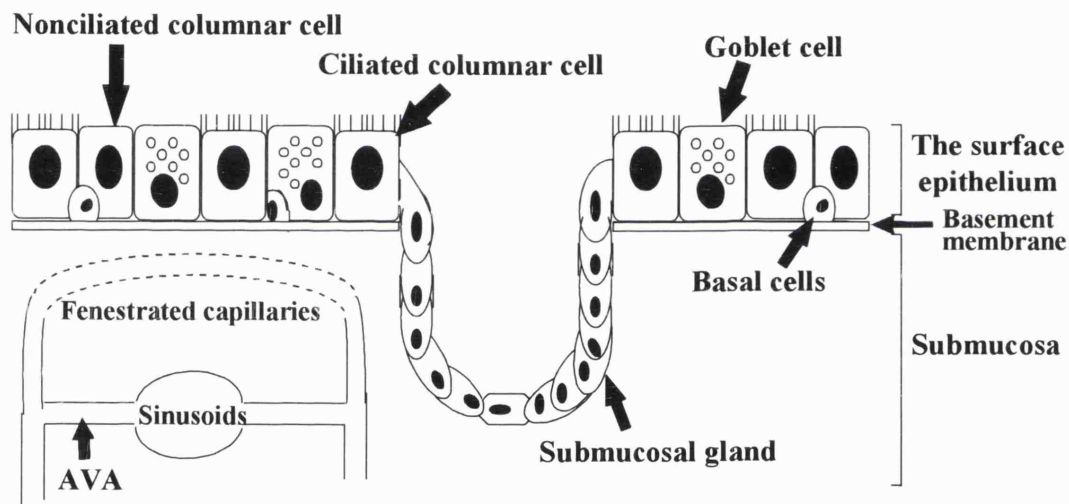


Figure 1.1: Schematic representation of human nasal mucosa (adapted from Naclerio *et al.*, 1993; Baraniuk *et al.*, 1995).

The epithelium consists of non-ciliated columnar cells, ciliated columnar cells, basal cells and goblet cells. One of the properties of columnar cells is the active transport of ions against a concentration gradient, and they therefore play a key role in regulating water transport. Goblet or mucous-secreting cells play an important role in host defence mechanisms since mucus traps particles, which are then removed by ciliary action. Basal cells contact the basement membrane and do not reach the airway lumen, and they help the adhesion of columnar cells to the basement membrane.

Submucosa is a loose connective tissue composed primarily of nerves, blood vessels, glands and extravascular cells.

The vascularisation of the nose is very profuse and is of considerable importance when considering both normal and abnormal conditions. The three major groups of nasal vasculature are capillaries, arteriovenous anastomoses (AVA) and venous erectile tissue (venous sinusoids with smooth muscle). The capillaries are located close to the epithelial surface. The capillaries have been well described by Cauna *et al.* (1972) who noted pores or fenestrations along the capillary surface facing the epithelium. A consequence of this fenestration is the facilitation of fluid transport through the vascular wall, which also makes the absorption of drugs easy and rapid. Arteriovenous anastomoses have been described in the nasal mucosa. These vessels are common in the skin of fingers, toes and ear lobes where they function in thermoregulation, and the shunt vessels found in the nasal mucosa may have a similar role. Warming of the inspired air requires a high mucosal blood flow and arteriovenous anastomoses have a role in air conditioning. While the large network of capillaries and arteriovenous anastomoses play an important role in facilitating heat exchange, the venous sinusoids which form a muscular venous erectile tissue account for the rapid changes in the thickness of nasal mucosa. The filling of the venous erectile tissue determines the state of congestion of the mucosa and this regulates the nasal resistance to air flow. The control of vascular tone involves nerves and inflammatory mediators.

1.1.3 The physiology:

Apart from the sense of smell the nose has many other functions. Inhaled air is processed as soon as it passes through the nasal passages which immediately filter, warm, and humidify it. As already noted, the entire nasal cavity is lined with mucous membrane full of blood vessels; heat from the blood warms the incoming air as it passes. Evaporation from the membrane acts as a humidifier, moistening the air. The nose is also extremely effective in filtering off particles. The inhaled air is first

filtered by the hair at the nasal entrance. Smaller particles are then trapped by the mucous blanket which covers the mucous membrane of the nasal cavity. The removal of trapped particles is facilitated by mucociliary transport. The particles adhere to the viscid mucus which is then transported by ciliary action in a backward direction into the nasal pharynx, and then swallowed, with the exception of the front portion of the inferior turbinates, where transport is anteriorly (Naclerio *et al.*, 1993). The mucous blanket not only effectively filters and removes very small particles, it also lubricates and protects the nasal mucosa, and most importantly, it also provides immune defense since its contents include antimicrobial substances, immunoglobulins, and other substances that play an important role in host defense.

1.2 HUMAN NASAL SECRETION

On the surface of nasal mucosa there is a secretory blanket that is thought to consist of two separable layers; the surface mucous or gel layer and a deeper aqueous or serous layer (Lippmann *et al.*, 1970; Naclerio *et al.*, 1993; Widdicombe *et al.*, 1982). The surface mucous or gel layer consists of a mucous glycoprotein (MGP) blanket where microorganisms and foreign particles are trapped and transported by mucociliary action. The mucous blanket is selective, as large particles never reach the mucous membrane, whereas small molecules do and are readily absorbed. The mucous glycoprotein is constantly removed and replaced by the newly secreted MGP. The deeper aqueous layer is the layer on which the mucus floats. This layer of fluid contains most of the aqueous proteins, many of which are derived from glands, blood vessels and extravascular cells in the submucosa. In contrast to the surface layer, this layer does not turn over quickly. The stability of this layer of fluid may provide many of the protective functions in host defense.

1.2.1 Major proteins in nasal secretion

Nasal secretion is composed of a complex mixture of compounds. The composition of this mixture and the volume of the secretion changes in response to exogenous stimuli. Baseline resting secretions include the following major proteins; MGP, albumin, immunoglobulin G (IgG), secretory immunoglobulin A (sIgA), lactoferrin and lysozyme. Nasal secretion and its constituent proteins are derived from; epithelial cells, submucosa glands, blood vessels and extravascular cells (Butler *et al.*, 1967; Naclerio *et al.*, 1993; Kaliner *et al.*, 1986; Widdicombe *et al.*, 1982).

Mucous glycoprotein is a macromolecule derived from two different sources; the goblet cells on the surface epithelium, and the submucosal gland mucous cells. Mucous cells are the major sources of MGP as they outnumber the goblet cells. MGP consists of a protein core onto which oligosaccharide side chains are attached. The oligosaccharide side chains are attached to the peptide region by O-glycosidic linkages between N-acetylglucosamine and threonine or serine (Kaliner *et al.*, 1986).

Submucosal glands contain mucous cells and serous cells. The serous cells do not produce MGP but produce bactericidal compounds, enzymes, lysozyme, lactoferrin and the secretory form of immunoglobulin A (Basbaum *et al.*, 1990; Verdugo *et al.*, 1990; Naclerio *et al.*, 1993). Lysozyme was one of the first proteins recognized as a product of the airway submucosal serous cells. It is a cationic protein with enzymatic activity that hydrolyzes the bacterial cell wall, thus killing the bacteria. Lactoferrin is another antimicrobial protein secreted by serous cells. It was first discovered in bovine and human milk and was later detected in many secretions protecting body surfaces, including bronchial, lachrymal and salivary. It is an iron-binding protein. Its antibacterial action may reduce the growth of iron-dependent bacteria through iron deprivation. Peroxidases are a family of enzymes present in serous cells. They catalyze the reduction of hydrogen peroxide to water by electron donors. In combination with hydrogen peroxide and thiocyanate, peroxidases are active against bacteria, viruses and fungi. Thus, they also play a role in the defense against infectious agents.

Immunoglobulin A (IgA) is one of the major immunoglobulins in nasal secretion. It is present in nasal secretion in two forms; monomeric IgA and sIgA (Widdicombe *et al.*, 1982; Kaliner *et al.*, 1991). sIgA is largely produced by interstitial plasma cells which are clustered around submucosal glands. The signals which stimulate plasma cells to cluster around submucosal glands and secrete dimeric IgA are not yet determined. The locally produced IgA is dimeric. Although the serous cells do not produce IgA, they synthesize the glycoprotein receptor generally known as the secretory component. Dimeric IgA binds to the secretory component and forms sIgA which is transported transcellularly through the serous cells into glandular secretions and becomes part of those glandular secretions (Widdicombe *et al.*, 1982). The source of monomeric IgA is the blood. Its presence in the baseline secretion is much less than sIgA but its concentration in the secretion can be higher following an increase in vascular permeability (Kalinier *et al.*, 1991; Raphael *et al.*, 1989b). IgA has viral neutralizing activity which could prevent colonization on the surface of the nasal mucosa.

Immunoglobulin G (IgG) is another immunoglobulin that is distributed in the nasal mucosa. It is a plasma protein that is found diffusely throughout the mucosa, but the highest concentration can be found near the basement membrane. Although IgG is present in relatively small proportions in baseline secretions (Kalinier *et al.*, 1991), it is found in much higher concentrations once vascular permeability has been increased. Another important plasma protein found in human nasal secretion is albumin. In resting secretion, albumin enters the nasal mucosa by passive transudation from blood vessels. It represents 2-10% of the total protein in resting secretion (Raphael *et al.*, 1988 and 1989b).

Table 1.1 Protein constituents of nasal secretions.

Source of protein	Protein
Mucous cells	Mucous glycoprotein (Kaliner <i>et al.</i> , 1986 and 1991)
Serous cells	Lactoferrin (Raphael <i>et al.</i> , 1989b and 1991) Lysozyme (Raphael <i>et al.</i> , 1989b and 1991) Secretory IgA (Kaliner <i>et al.</i> , 1991; Widdicombe <i>et al.</i> , 1982) Aminopeptidase (Raphael <i>et al.</i> , 1991) Peroxidase (Kaliner <i>et al.</i> , 1991)
Plasma origin	Albumin (Raphael <i>et al.</i> , 1988 and 1989b) IgG (Kaliner <i>et al.</i> , 1991; Raphael <i>et al.</i> , 1991) monomeric IgA (Kaliner <i>et al.</i> , 1991; Widdicombe <i>et al.</i> , 1982) IgM (Kaliner <i>et al.</i> , 1991; Raphael <i>et al.</i> , 1991) IgE (Raphael <i>et al.</i> , 1991) carboxypeptidase N (Kaliner <i>et al.</i> , 1991; Proud <i>et al.</i> , 1987) angiotensin-converting enzyme (Proud <i>et al.</i> , 1987)

1.3 ALLERGIC RHINITIS

Rhinitis is the term used to imply an inflammation of the nasal mucous membrane. Discomfort, itching, sneezing, rhinorrhea (runny nose) and reduced patency (nasal blockage) are classical symptoms of rhinitis. Rhinitis may be classified as allergic or non-allergic. The symptoms of rhinitis can be triggered by exposure to antigens in allergic rhinitis, or by a variety of stimuli such as viral infection and certain drugs for the non-allergic forms. Allergic rhinitis is classified as a type 1 hypersensitivity reaction. Hypersensitivity is the term applied when an adaptive immune response occurs in an exaggerated or inappropriate form causing a release of mediators which produce an inflammatory reaction. This response is not a general one but is characteristic of an individual (atopic) who has the tendency to react to allergen such as grass pollen or products of house dust mites. The development of sensitivity to an

offending allergen requires the synthesis of IgE antibodies directed at the antigenic epitope. This development of sensitivity involves a stage of sensitisation when inhaled allergen enters via a mucosal surface and is taken up by local antigen-presenting cells which process and present them to T-helper cells. The antigen is also recognised by B lymphocytes. The T-helper cells then release soluble factors (e.g. IL-4) which induce B cells to proliferate and differentiate, which in turn leads to a production of allergen-specific IgE. The IgE then binds, via Fc_ϵ receptors to mast cells thus sensitizing the mast cells. The second stage involves subsequent exposure to the allergen which then reaches the sensitized mast cell; it cross-links surface-bound IgE causing a release of pre-formed mediators such as histamine and proteases and newly synthesized lipid-derived mediators such as PGD_2 , LTC_4 and PAF. The mediators released act on specific target sites to produce the clinical effect of allergy.

The allergic rhinitis can be divided into seasonal and perennial. Seasonal allergic rhinitis, for which the generally accepted term is hay fever, is determined by short exposure to inhaled allergens such as tree pollen and grass pollen during the springtime. In perennial allergic rhinitis, on the other hand, the exposure to the allergens occurs all year round. Perennial allergic rhinitis may thus be more difficult to diagnose than seasonal allergic rhinitis. House-dust mite is one of the most common allergens in perennial allergic rhinitis. Non-allergic rhinitis is either infectious or non-infectious rhinitis. Vasomotor rhinitis is a classical example of non-infectious rhinitis, and can be mimicked by eating hot/spicy foods such as hot chilli peppers (Raphael *et al.*, 1989c). Infectious rhinitis can be subdivided into acute and chronic forms. The latter may be due to infection with specific organisms such as syphilis, tuberculosis or rhinoscleroma leprosy. The acute infectious rhinitis can be caused by bacteria or viruses. Most people have experienced naturally-induced acute infectious rhinitis in the form of the common cold (Mygind *et al.*, 1993).

The major pathological changes which occur in allergic rhinitis are nasal airway inflammation and hyperresponsiveness. Nasal blockage and rhinorrhea are an indication of an inflammation of the mucosal lining of the nose. Mediators such as histamine and bradykinin act directly on blood vessels and submucosal glands,

causing vasodilatation, increased mucosal vascular permeability with oedema and glandular secretion, leading to nasal blockage. Glandular secretion together with leakage of plasma fluid causes rhinorrhea. Histamine and bradykinin may excite sensory nerve endings in the nasal epithelium and deeper mucosal tissues setting up axon reflexes with the release of neuropeptides, such as substance P, neurokinin A and calcitonin gene related peptide (CGRP). These neuropeptides cause vasodilatation and transudation, and may modulate the secretions from submucosal glands (Cauna *et al.*, 1972). Activation of the same sensory nerves also initiates central nervous reflexes via the sympathetic and parasympathetic motor nerves. The responses include itching and sneezing and changes in the nasal vasculature and secretion.

Airway hyperresponsiveness has been stressed during the last decade as one of the major factors involved in the pathogenesis of allergic airways disease. Nasal hyperresponsiveness can be described as a clinical feature characterized by the increase in responsiveness to daily life stimuli, such as dust and fumes. More than 80% of patients with perennial rhinitis find that these nonspecific stimuli provoke symptoms (Kirkegaard *et al.*, 1987). Several hypotheses for the mechanism of nasal hyperresponsiveness have been suggested. These include epithelial damage or malfunction, leading to increased access of agents to the site of action; increased sensitivity of sensory nerve endings; changes in parasympathetic and/or sympathetic tones (David & Devalia, 1995); upregulation of receptors, e.g. the number of muscarinic receptors has been found to increase with nasal allergy (Konno *et al.*, 1987b); and redistribution towards the mucosal surface or increase in the number of inflammatory cells. To date there is no evidence that allergic reactions in the nose cause a histological destruction of the surface epithelium similar to that suggested in the lower airway. However, this does not exclude a functional disturbance of the nasal epithelial lining which may result in increased exposure to target sites such as neurones and inflammatory cells. An increase in the number of eosinophils on and in the nasal mucosa has been observed during the allergy season (Iliopoulos *et al.*, 1990; Juliusson *et al.*, 1992). Increases in mast cell numbers have also been found during the allergy season (Viegas *et al.*, 1987a and b; Enerbäck *et al.*, 1986).

The initial response to allergen exposure in allergic rhinitis is known as the early-phase response. However, nasal late-phase responses which develop 2 to 12 hours following the exposure may occur. This late-phase response is characterised by a prolonged nasal congestion, influx of inflammatory cells and mediators, which derive from a variety of resident and emigrating cells, and nasal hyperresponsiveness to antigen challenge.

1.4 INFLAMMATORY CELLS IN HUMAN NASAL MUCOSA

1.4.1 Mast cells and basophils

Mast cells and basophils were first described by Paul Ehrlich more than 100 years ago as cells that had prominent cytoplasmic granules that could be stained with basic dyes. Basophils are found in small numbers in the circulation (less than 0.2% of leucocytes). The mast cell is not found at all in the circulation, and is resident only in body tissues. Mast cells and basophils are derived from a pluripotential stem cell in the bone marrow. Basophils complete their differentiation in the bone marrow, then enter the circulation and can migrate into tissues at the sites of inflammation, particularly during that late phase of allergic reactions. Mast cell precursors, on the other hand, leave the bone marrow via the blood and complete their differentiation in the tissues. There are two types of human mast cells, which may be classified according to the proteases present; the mucosal types which are tryptase + (MC_T) and the connective tissue type which are tryptase+ and chymase+ (MC_{TC}). MC_T are found predominantly in the epithelium whereas MC_{TC} are found predominantly in the submucosa.

In nasal mucosa, most mast cells are found in the superficial 200 μm , generally clustered just beneath the basement membrane, and in close proximity to the nerves and blood vessels. In normal subjects, there are about 7000 mast cells/mm³ in the submucosa but only 50/mm³ in the epithelium (Friedman & Kaliner, 1985; Kaliner *et al.*, 1994). It has been suggested that in allergic rhinitis, exposure to pollen leads to

migration of the connective tissue cell types into the epithelium, where they differentiate into the mucosal type, under the influence of cytokines, derived possibly from T-helper cells or epithelial cells (Kawabori *et al.*, 1985; Denburg *et al.*, 1989). The number of mast cells increases during the pollen season in patients with seasonal allergies (Viegas *et al.*, 1987a and 1987b).

Upon activation, mast cells release preformed mediators, cytokines and synthesize eicosanoids from arachidonic acid. Histamine is a major preformed mediator that is released. It has long been recognised for its involvement in acute allergic reactions and was one of the first chemical substances to be associated with mast cells. A number of other compounds which are chemotactic factors for eosinophils, neutrophils and monocytes are also present. Prostaglandin D₂ (PGD₂) is the main product of arachidonic acid in mast cells, although leukotriene C₄ (LTC₄) is also produced, but in smaller amounts compared to PGD₂. When activated, basophils also release histamine and LTC₄, but not PGD₂. Thus, the absence of PGD₂ in the presence of LTC₄ and histamine can be used to implicate basophil activation (Naclerio *et al.*, 1985a).

Basophils are thought to be responsible for the release of histamine in the late-phase response following allergen challenge, as there is no release of PGD₂ (Naclerio *et al.*, 1985a). This implication is supported by the finding that the number of basophils in the nasal mucosa is increased following nasal grass-pollen challenge in seasonal allergic subjects. Also pretreatment with corticosteroids inhibits the release of histamine in the late response but not in the early response (Bascom *et al.*, 1988b), and corticosteroids have been shown to inhibit mediator release from basophils (Schleimer *et al.*, 1981) but not mast cells (Schleimer *et al.*, 1983).

Therefore, mast cells and basophils can release mediators which are capable of producing the clinical effects of rhinitis. Mast cells are the major source of histamine, which is responsible for the immediate response to allergen, while basophils are the major source of histamine and may play a role in the late response to allergen.

1.4.2 Eosinophils

Eosinophils are associated with allergic diseases. Many studies have shown that there is a close association between inflammation, epithelial damage and hyperresponsiveness (Devalia *et al.*, 1992; Smith *et al.*, 1992; Oddera *et al.*, 1996), and that eosinophils may be the effector cells of epithelial damage in asthmatic subjects (Gleich *et al.*, 1979; Ayars *et al.*, 1989; Hastie *et al.*, 1987; Motojima *et al.*, 1989). There is now increasing evidence to suggest that eosinophils may also be associated with allergic rhinitis. Eosinophils are readily detectable in nasal secretions following exposure to allergen, and their numbers correlate well with the occurrence and severity of symptoms of rhinitis during the pollen season (Pipkorn *et al.*, 1988).

On activation, eosinophils release a number of mediators including major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) which are stored in granules. Other mediators such as platelet-activating factor (PAF), leukotriene C₄ (LTC₄), oxygen metabolites and cytokines are generated following eosinophil activation. PAF has been implicated as a mediator of bronchial hyperresponsiveness (Cuss *et al.*, 1986). It is also a potent chemotactic agent for eosinophils (Hencoq & Vargaftig, 1986; Klementsson & Andersson, 1992) and is important as one of the mediators of nasal hyperresponsiveness (Austin & Foreman, 1993). ECP may play a role in stimulating glandular secretion since it induces secretion from feline tracheal explants (Lundgren & Shelhamer, 1990b). MBP has been demonstrated to stimulate basophil histamine release (O'Donnell *et al.*, 1983; Zheutlin *et al.*, 1984) and cause epithelial injury in the asthmatic. MBP and ECP are increased in the nasal lavage of subjects with perennial and seasonal allergic rhinitis (Linder *et al.*, 1987; Togias *et al.*, 1988; Bascom *et al.*, 1989). The amount of MBP present during the late response correlates closely with the numbers of eosinophils (Bascom *et al.*, 1989) and these findings provide evidence for degranulation of eosinophils, and their participation in the late response to allergen. Pretreatment with oral corticosteroids causes a dramatic decrease in the amount of MBP and EDN during the late response. The major factor accounting for this is, most likely, the decrease in eosinophil influx caused by oral corticosteroid pretreatment (Bascom *et*

al., 1988a). Eosinophils may play an important role in the pathogenesis of allergic rhinitis and particularly during the late-phase response because of the potent mediators they release.

1.4.3 Neutrophils

Neutrophils are the most numerous white cells in the blood. They possess two main types of granules; the primary (azurophilic) granule and the secondary or specific granule. Upon activation of neutrophils, the cells degranulate, secreting the contents of various granules. The principal bactericidal activity of neutrophils is the oxidase system contained within azurophilic granules. Myeloperoxidase (MPO) of the azurophilic granules reacts with hydrogen peroxide and chloride to generate hypochlorous acid, a lethal oxidizing agent and potent killer of microorganisms. Furthermore neutrophils are also capable of synthesizing LTB₄, LTC₄ and PAF (Barnes & Costello, 1987).

Neutrophils have been identified in nasal lavage in increased numbers following allergen challenge (Bascom *et al.*, 1988a). In both *in vitro* and *in vivo* studies, there is a release of high-molecular-weight neutrophil chemotactic activity (HMW-NCA) from resected human turbinate membranes of allergic rhinitic subjects and in nasal secretions from patients with allergic rhinitis, after antigen challenge (Nagakura *et al.*, 1989; Mackay *et al.*, 1986). However, a nasal allergen challenge study using MPO as a marker of neutrophil activity showed no relationship between MPO and the symptoms of the patients (Linder *et al.*, 1987).

To date there is no evidence to suggest that neutrophils are important in the pathogenesis of allergic rhinitis. Despite the lack of evidence, neutrophils may contribute to the pathogenesis of nasal hyperresponsiveness since the contents of neutrophils could be damaging or disruptive to epithelial cells if neutrophils are inappropriately activated.

1.4.4 Epithelium

Airway epithelium (which has been regarded as a physical barrier, preventing the entry of inhaled foreign particles into the submucosa) may play a role in the aetiology of allergic rhinitis. Epithelial cells can generate and release specific inflammatory mediators which are potent chemoattractants, activators of inflammatory cells and cytokines which have profound effects on growth, differentiation, migration and activation of inflammatory cells. Epithelial cells can express specific inflammatory cell-adhesion molecules which play a role in inflammatory cell migration.

Human airway epithelial cells are capable of metabolizing arachidonic acid to prostaglandins, leukotrienes and possibly platelet-activating factor. The generation of these mediators and their relevance to alteration of airway function and inflammation will be discussed later. Airway epithelial cells and cell lines are capable of synthesizing cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 1 (IL-1) and tumor necrosis factor- α (TNF α) (Spriggs *et al.*, 1988; Ohtoshi *et al.*, 1991, Kenney *et al.*, 1994). An *in vitro* study has demonstrated that GM-CSF enhances the viability and functional activation of eosinophils (Owen & Crow, 1987). Epithelial cells in patients with allergic rhinitis synthesized significantly more GM-CSF than the cells of the nonatopic nonrhinitic group (Ohtoshi *et al.*, 1991). TNF α has been demonstrated to increase epithelial permeability (Mullin & Snock, 1990) and it is also active in the upregulation and expression of IL-8 (a major neutrophil chemotactic factor) (Kunkel *et al.*, 1990) and intercellular adhesion molecule 1 (ICAM-1). ICAM-1 has been reported to be expressed in the nasal epithelium of allergic rhinitics (Altman *et al.*, 1993; Devalia & Davies, 1991). ICAM-1 is active in the recruitment and migration of neutrophils and eosinophils (Wegner *et al.*, 1990) and may play a role in the trafficking of these inflammatory cells to the epithelium.

Airway epithelial damage is a critical feature of airway hyperresponsiveness in the asthmatic. Epithelial shedding is a consequence of the action of substances, such as eosinophil MBP and oxygen-derived free radicals, together with various proteases

released from inflammatory cells. In asthma, epithelial shedding may remove a protective barrier, thus allowing allergens and inhaled chemicals to reach cells in the submucosa. To date there is no experimental evidence that such a destruction of the epithelium, as in bronchial epithelium, occurs in nasal epithelium. A study of nasal epithelium in allergic and non-allergic subjects shows that the epithelium tissue of perennial rhinitics is significantly thickened as compared to that in the tissue of either normal or seasonal allergic rhinitic subjects (Calderon *et al.*, 1994). The difference in the thickness between the epithelium of seasonal allergic rhinitics and perennial allergic rhinitics may be a result of the duration of the exposure to allergens. The thickening of the epithelium could be a self-preservation effect against any damage which may occur from prolonged exposure to allergens, as in the case of perennial allergic rhinitis. The thickening of the epithelium and the consequent narrowing of the airway of the nasal cavity may contribute to increases in nasal resistance. It is possible that in chronic allergic rhinitis, dysfunction or damage of the nasal epithelium may occur which may lead to an increased generation of mediators, cytokines and expression of adhesion molecules.

1.4.5 Lymphocytes and Langerhan's cells

In addition to the inflammatory cells already mentioned, lymphocytes and Langerhan's cells may also contribute to nasal inflammation. Lymphocytes and Langerhan's cells are found in large numbers in the nasal mucosa of allergic rhinitic subjects (Fokkens *et al.*, 1990). It is not entirely clear how these cells are involved in the pathophysiology of allergic rhinitis, but it is likely that they are involved in immune responses. T cells may play a modulatory role since they have been shown to release cytokines which influence both the migration and activity of inflammatory cells. Langerhan's cells are known to be important antigen-presenting cells and may activate T cells.

1.5 MEDIATORS AND NEUROTRANSMITTER IN HUMAN NASAL MUCOSA

1.5.1 HISTAMINE

Histamine (β -imidazolyl-ethylamine) has long been recognized to be a mediator of allergic and inflammatory reactions in humans, and is one of the chemical substances contained within mast cells. Sir Henry Dale demonstrated the potent bronchospastic and vasodilator activity of histamine when injected intravenously into animals (Dale *et al.*, 1911). Many of the symptoms of antigen injection into a sensitized animal could be reproduced by histamine. An injection of histamine into skin produced the triple response (erythema, flare and wheal) signs that characterized the response observed in injured skin (Olsson *et al.*, 1988). Histamine was, therefore, considered to be a mediator of the acute allergic response, since it mimicked allergic and inflammatory reactions.

1.5.1.a Synthesis and catabolism of histamine

The major sites of histamine storage and release are mast cells. Other sites include basophils and neurons in the CNS (Riley *et al.*, 1953; Arrang *et al.*, 1983). Histamine is synthesized in the Golgi apparatus of mast cells and basophils by decarboxylation of histidine, figure. 1.2. Once formed, it is found in ionic association with the acidic side chains of heparin or a related proteoglycan (Riley *et al.*, 1953; Wood-Baker *et al.*, 1995).

Histamine is secreted following cell activation with stimuli, including antigen, anti-human IgE, concanavalin A, substance P, opiates, compound 48/80 and a range of lymphokines and cytokines. The mast cell subpopulations and basophils may respond differently to these stimuli. Upon stimulation, histamine is released from the cells by a secretory process involving rapid dissociation of histamine from the partially solubilized granule matrix by exchanging with sodium ions in the matrix

environment. Histamine is rapidly metabolized by either of two enzymatic pathways; methylation by methyltransferase (70%) and oxidation by diamine oxidase (30%). Neither enzyme is histamine specific and they are widely distributed in tissues (Wood-Baker *et al.*, 1995).

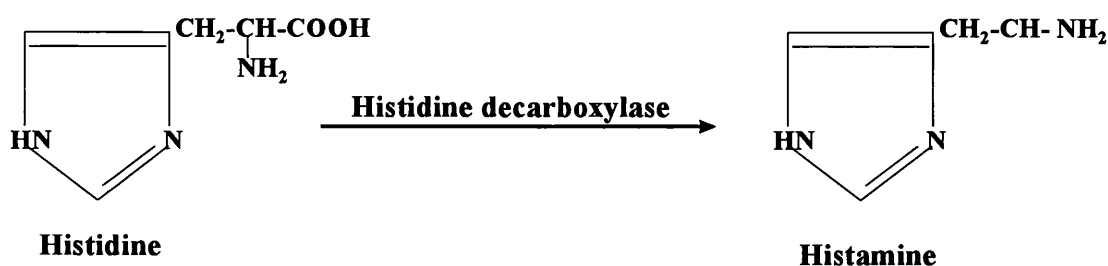


Figure 1.2: Formation of histamine (Wood-Baker *et al.*, 1995)

1.5.1.b Histamine receptors and their biological activity

Histamine has wide ranging biological activities mediated through specific histamine receptors, which are of three main types; H₁, H₂ and H₃, distinguished by their selective antagonists (Wood-Baker *et al.*, 1995; Hill *et al.*, 1997). The role of H₃ receptor activation is not fully established at present as compared to the first two types. However H₃ receptors may be associated with modulation of cholinergic and sensory nerves (Arrang *et al.*, 1983; Hill *et al.*, 1997). Recently another histamine receptor subtype, H₄, has been identified (Nakamura *et al.*, 2000; Oda *et al.*, 2000). The H₄ receptor is more closely related to the H₃ receptor than the H₁ or H₂ receptor. The H₄ receptor has been reported to be expressed on a variety of cell types, including peripheral blood mononuclear cells, neutrophils, eosinophils, mast cells and resting CD4⁺ cells (Nakamura *et al.*, 2000; Oda *et al.*, 2000; Nguyen *et al.*, 2001; Liu *et al.*, 2001; Zhu *et al.*, 2001). The distribution of H₄ receptors suggests that the H₄ receptor may play an important role in immune function and the pathogenesis of allergic rhinitis. The main action of histamine on H₂ receptors is the secretion of gastric acid (Black *et al.*, 1972). H₁ receptors are found in bronchial muscle and upon activation

by histamine induce bronchoconstriction. Histamine also induces vasodilatation and vascular permeability via activation of H₁ receptors (Ash & Schild, 1966).

The effects of histamine on the vascular system are complex, since different vascular beds show different responses. However most vascular beds respond with vasodilatation, mediated by either or both H₁ and H₂ receptors (Braude *et al.*, 1984; Wood-Baker *et al.*, 1995). H₁ receptors mediate the release of PGI₂ and endothelium-derived relaxing factor (EDRF), also known as nitric oxide, by the vascular endothelium (Palmer *et al.*, 1987). These produce relaxation of the underlying smooth muscle and hence vasodilatation. Histamine induces vascular permeability through vasodilatation together with contraction of endothelial cells upon binding to H₁ receptors on the cells. The contraction of the cells allow fluid and plasma protein to leak into surrounding areas.

The main action of histamine on the vascular system can be observed in the triple response that follows intradermal injection of histamine (Ash & Schild, 1966). The blood vessels immediately affected by the histamine dilate and produce a local erythema. Then, the surrounding blood vessels dilate and a flare is produced. The flare is due to an axon reflex, as the histamine stimulates sensory nerves which send impulses orthodromically and then antidromically down the branches of the sensory nerve fibres. The orthodromic impulses give rise to the sensation of itching and pain or a burning sensation, depending on the concentration. The antidromic impulses produce the secondary dilatation which is mediated by neuropeptides released from the sensory nerve endings supplying the blood vessels. A wheal that appears at the site of injection, results from oedema formation. The local erythema results from the direct action of histamine on both H₁ and H₂ receptors, with the major contribution by the H₁ component (Foreman *et al.*, 1987(a), (b), (c) and 1988).

1.5.1.c Histamine as a mediator of rhinitis

It is important to understand the action of histamine in the nose since it is the major mediator of acute allergic reactions. Histamine is not only found in nasal lavage immediately following allergen challenge (Naclerio *et al.*, 1983a and 1983b) but also in the lavage fluid 2-6 hours following the challenge (Naclerio *et al.*, 1985a). As already mentioned, mast cells are the major source of histamine in the early response whereas the presence of histamine during the late response is of basophilic origin. The actions of histamine in allergic rhinitis have been studied *in vivo* by evaluation of the response to nasal provocation with histamine and the effect of H₁ receptor antagonists on nasal challenge with histamine or allergen.

Intranasal challenge with histamine generates symptoms characteristic of rhinitis, with itching, sneezing, rhinorrhea and congestion. The nasal effects of histamine are primarily mediated via the H₁ receptor (Rokenes *et al.*, 1988; Simons & Simons, 1988). H₁ antagonists effectively treat allergic rhinitis during the immediate reaction to allergen by reducing itching, sneezing and rhinorrhea (Bousquet *et al.*, 1988; Naclerio *et al.*, 1989; Naclerio & Togias, 1991) but have a lesser effect on nasal congestion. The combination of H₁ and H₂ antagonists administered orally has been reported to be more effective than H₁ antagonists alone in inhibiting nasal congestion induced by histamine provocation (Secher *et al.*, 1982). In contrast, the combination had no effect on nasal airway resistance when applied topically (Holmberg *et al.*, 1989). The inability of anti-histamines to eliminate all the symptoms of allergic rhinitis indicates the possibility that other mediators may be involved and possibly that histamine has an indirect action on the nasal mucosa.

1.5.2 METABOLITES OF ARACHIDONIC ACID

Arachidonic acid is a membrane associated polyunsaturated fatty acid that is nearly ubiquitous in mammalian cells. Enzymatic oxidation of this fatty acid produces a variety of metabolites that have important biochemical, cellular and physiological

roles. Among the metabolites are the prostaglandins, leukotrienes, thromboxane, and prostacyclin. The principle sources of arachidonic acid are membrane bound phospholipids. Arachidonic acid is released into the cell via hydrolysis of phospholipids by phospholipase A₂, phospholipase C and diglyceride lipase. Free arachidonic acid may be primarily enzymatically oxygenated by cyclo-oxygenase and lipoxygenase.

1.5.2.1 PROSTANOIDS

1.5.2.1.a Synthesis and catabolism of prostanoids

Cyclo-oxygenase (COX) exists in two forms; COX-1 and COX-2. COX-1 is found in most cells as a constitutive enzyme whereas COX-2 formation is induced in inflammatory cells. COX is located in endoplasmic reticulum and nuclear membranes and catalyzes the oxidation of arachidonic acid to prostaglandins, thromboxane and prostacyclin. Prostaglandin D₂ (PGD₂), prostaglandin E₂ (PGE₂), prostaglandin F_{2α} (PGF_{2α}), prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) are the most important products of the COX pathway. The products of arachidonic acid metabolites vary in different cells. In platelets, the pathway leads to TXA₂ synthesis, in vascular endothelium it leads to prostacyclin synthesis and in macrophages it leads mainly to PGE₂. As already noted, mast cells synthesize PGD₂. The products of COX are regarded as local hormones as they usually act close to their sites of production.

Several intracellular enzymes are involved in the inactivation of prostanoids. Prostaglandins of the E and F series are rapidly metabolized by sequential oxidation and reduction, by prostaglandin 15-hydroxydehydrogenase (PGDH) and delta-reductase (delta-R) respectively, to yield inactive metabolites. PGD₂ on the other hand is reduced to yield a product that has similar characteristics to F-series prostaglandins before being inactivated by PGDH/delta-R pathways. In the case of TXA₂ and PGI₂, they are rapidly hydrolyzed to much less active substances TXB₂ and 6-oxo-PGF_{1α}.

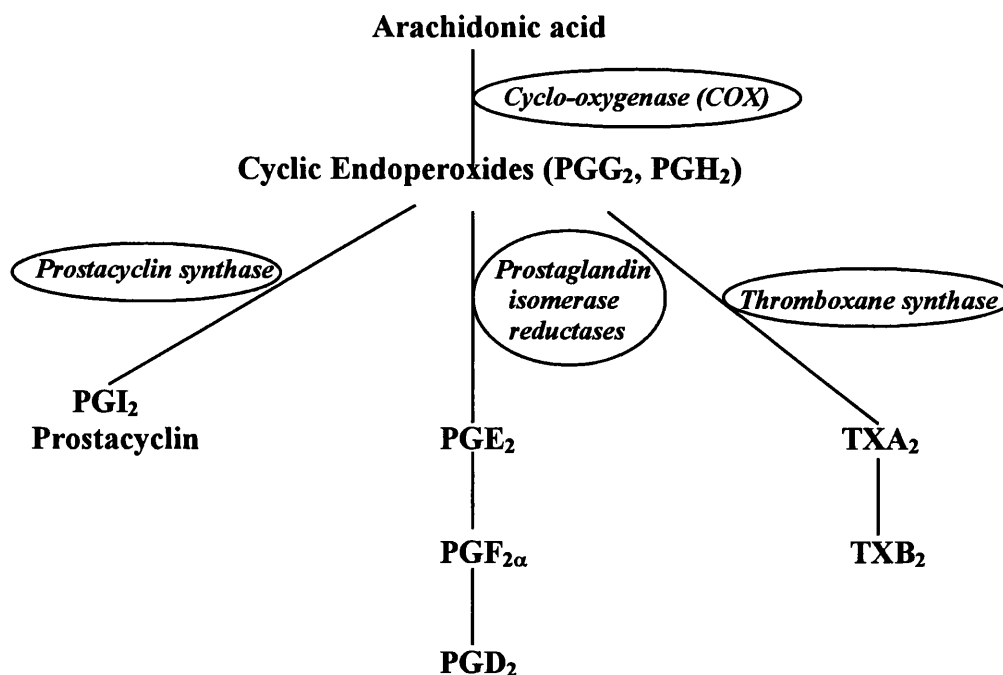


Figure 1.3: Synthesis of prostanoinds (Moncada *et al.*, 1979; Vane *et al.*, 1982; Kleeberger *et al.*, 1995).

1.5.2.1.b Receptors and the biological action of prostanoinds

The characterization of prostanoind receptors is a developing field of investigation which is hampered by a lack of potent selective antagonists. Presently, prostanoind receptors have been classified into five types, based on the potencies of natural prostanoinds and competitive antagonists, and on identification of ligand binding sites using radiolabelled agonists or antagonists (Gardiner *et al.*, 1990; Halushka *et al.*, 1986). The receptor types are: DP, EP, FP, IP and TP. One receptor type is for each prostanoind, DP for PGD₂, EP for PGE₂, FP for PGF₂, IP for PGI₂ and TP for TXA₂. There is more than one EP receptor.

PGD₂, PGE₂ and PGI₂ are powerful vasodilators. PGD₂ injected intradermally into human skin causes immediate wheal and flare, which is accompanied by neutrophil infiltration (Flower *et al.*, 1976b; William *et al.*, 1977). PGD₂ is a chemoattractant of human neutrophils and it augments the leukotriene B₄-mediated accumulation of neutrophils in human skin (Soter *et al.*, 1983). PGD₂ potentiates the

bronchoconstrictor response to both histamine and methacholine in asthmatic subjects (Fuller *et al.*, 1986). At the same time, PGD₂ and TXA₂ are potent bronchoconstrictors in their own right. In addition to bronchoconstrictive properties, TXA₂ has been reported to stimulate airway smooth muscle cell proliferation (Noveral & Grunstein, 1992). Also PGE₂ and PGI₂ have relaxant effects on airway smooth muscle. PGE₂ and PGI₂ inhibit platelet aggregation whereas TXA₂ induces platelet aggregation. In addition to broncho- and vasoactive effects, prostanoids are involved in the modulation of neuronal activity and vascular tone and permeability (Beasley *et al.*, 1987). They potentiate the increase in vascular permeability produced by histamine and bradykinin (Flower *et al.*, 1976b; William *et al.*, 1977).

1.5.2.1.c Prostanoids and rhinitis

Numerous studies have reported the release of prostanoids into nasal secretions after an allergen challenge in subjects with allergic rhinitis (Naclerio *et al.*, 1983a and 1983b; Brown *et al.*, 1987; Ramis *et al.*, 1991). Among the five main prostanoids, PGD₂ is quantitatively the dominant prostanoid in allergic challenge, having been generated in large amounts by mast cells (Holgate *et al.*, 1984; Lewis *et al.*, 1982). Many studies show an elevation of PGD₂ concentration following nasal allergen challenge (Naclerio *et al.*, 1983a; Norman *et al.*, 1985; Togias *et al.*, 1985a; Browns *et al.*, 1987; Pipkorn *et al.*, 1987).

PGD₂ is only present in nasal lavage recovered during the immediate response but not during the late response (Naclerio *et al.*, 1985a; Norman *et al.*, 1985). Increased concentrations of PGD₂, leukotrienes and a concomitant infiltration of inflammatory cells were reported to occur within minutes of the allergen-induced nasal response (Georgitis *et al.*, 1991). As indicated previously, the pattern of histamine elevation during the immediate and late response and elevations of PGD₂ only during the immediate response, have suggested that basophils are recruited to the nose and participate in the late phase response of allergic rhinitis. Nasal provocation with PGD₂ induced an increase in nasal airway resistance without inducing sneezing or rhinorrhea (Howarth *et al.*, 1991). PGD₂ has been shown to be more effective in

producing nasal congestion than either histamine, bradykinin or methacholine, and it produces symptoms of rhinorrhea and sore throat (Doyle *et al.*, 1990).

Pretreatment with aspirin, a COX inhibitor, prior to a nasal allergen challenge reduced the generation of prostaglandins without changing the anticipated increases in histamine and leukotrienes during the early response. However, aspirin has no effect on the symptoms scores record after the challenge (Naclerio *et al.*, 1985a; Brown *et al.*, 1987). Flurbiprofen, another COX inhibitor, had a significant effect on reducing secretion, the number of sneezes and overall subjective severity score during the immediate nasal reaction (Brooks *et al.*, 1984). However, nasal airway resistance was not significantly affected. The lack of efficacy of COX inhibitors in reducing nasal blockage suggests that prostanoids may not be the primary mediators of allergic inflammation. The ability of flurbiprofen to reduce some symptoms of allergic rhinitis while aspirin failed, could be due to the fact that aspirin selectively inhibits COX-1 whereas flurbiprofen selectively exerts its effect on COX-2 (Meade *et al.*, 1993). This would suggest that the generation of prostanoids from inflammatory cells by COX-2 may play a role in inducing symptoms other than nasal congestion.

1.5.2.2 LEUKOTRIENES

1.5.2.2.a Synthesis and catabolism of leukotrienes

Leukotrienes are biologically active fatty acids derived from the oxidative metabolism of arachidonic acid. They consist of the cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄) and the noncysteinyl leukotriene (LTB₄). After arachidonic acid is cleaved from membrane phospholipids, the next step in the formation of leukotrienes can proceed. LTA₄, a precursor for both LTB₄ and cysteinyl leukotrienes, is formed via the intermediate 5-hydroperoxyeicosatetraenoic acid or 5-HPETE by the action of 5-lipoxygenase. 5-lipoxygenase is the main enzyme of the lipoxygenase group. It is located in the cytosol and is found in lung, platelets, mast cells and white blood cells. 5-lipoxygenation of arachidonic acid requires the presence of a membrane protein

known as the 5-lipoxygenase activating protein (FLAP), in order to proceed. Once formed, LTA₄ is immediately converted enzymatically by a hydrolase or glutathione-S transferase to LTB₄ or LTC₄, respectively. LTC₄ is specifically transported from the intracellular microenvironment to the extracellular milieu where it is converted sequentially to LTD₄ and LTE₄. LTC₄ is converted to LTD₄ by γ -glutamyl transpeptidase. LTD₄ is then converted by a dipeptidase to form LTE₄, figure 1.4.

LTB₄ is produced mainly by neutrophils while the cysteinyl leukotrienes are produced mainly by eosinophils, mast cells, basophils and macrophages, since these cells have the full enzymatic system to produce and export LTC₄. However, through the co-operative action of two distinct cell types, the formation of cysteinyl leukotrienes can also be possible. For example, in the lung, polymorphonuclear leukocytes are known to serve as LTA₄ donors, while vascular endothelial cells and platelets can transform the LTA₄ they receive from other cells to LTC₄ (Macclouf & Murphy, 1988; Feinmark & Cannon 1986, 1987a and 1987b).

Cysteinyl leukotrienes are degraded by three major pathways. The first pathway involves hydroxylation and carboxylation of LTE₄ with the formation of five different metabolites, two of which represent the major metabolites that can be recovered from the urine of humans after intravenous administration of LTE₄. The second degradation pathway leads to the formation of *N*-acetyl-LTE₄. The third pathway requires an inflammatory microenvironment, such as that which exists in the presence of activated polymorphonuclear leukocytes, to produce hypochlorous acid through the action of myeloperoxidase. The third pathway leads to the formation of diastereomeric 6-trans-LTB₄. LTB₄ can be converted to 20-hydroxy-LTB₄ by a unique membrane-bound P-450 enzyme that occurs in the neutrophil. It is then further oxidised to 20-carboxy-LTB₄. Although the biological activity of all these metabolites is not known, it is possible that the formation of each leukotriene metabolite is associated with a loss in bioactivity.

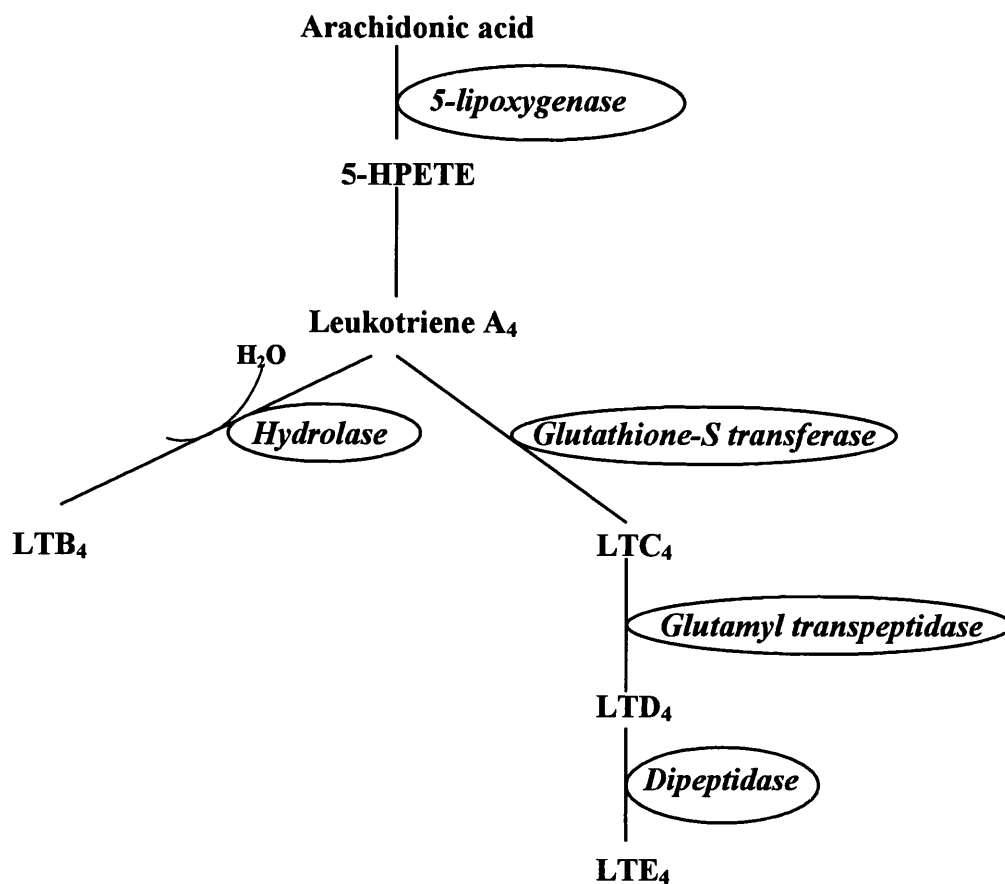


Figure 1.4: Synthesis of leukotrienes (Vane *et al.*, 1982; Drazen *et al.*, 1995).

1.5.2.2.b Receptors and the biological action of leukotrienes

There are receptors specific for LTB₄, LTC₄ and LTD₄ defined by selective agonists and antagonists. Whether there is a specific receptor for LTE₄ is not yet known, however LTE₄ appears to act as a partial agonist at the LTD₄ receptor (Saussy *et al.*, 1989).

LTB₄ is a powerful chemotatic agent for both neutrophils and macrophages. In neutrophils, it also enhances the neutrophil's ability to bind to endothelial cells (Nohgawa *et al.*, 1997) and increases the production of toxic oxygen products and granule secretion. In humans, subcutaneous injection of LTB₄ elicits a transient wheal

and flare, followed 4 to 6 hours later by a tender, indurated lesion, that contains fibrin, which is infiltrated with neutrophils (Soter *et al.*, 1983). LTC₄, LTD₄ and LTE₄ also induce a wheal that persists more than 2 hours along with a more persistent erythema when injected intradermally. Cysteinyl leukotrienes have a potent contractile action on bronchial smooth muscle and coronary and pulmonary vascular smooth muscle (Piper *et al.*, 1984; Piper & Samhoun, 1987). They also enhance vascular permeability via endothelial cell contraction and cause bronchial mucus secretion (Drazen *et al.*, 1980; Lewis *et al.*, 1990). Both LTD₄ and LTE₄ have been reported to increase airway hyperresponsiveness (Arm *et al.*, 1988).

1.5.2.2.c Leukotrienes and rhinitis

Cysteinyl leukotrienes (Creticos *et al.*, 1984; Miadonna *et al.*, 1987; Naclerio *et al.*, 1991b) and LTB₄ (Shaw *et al.*, 1985) have been recovered in increased amounts from the nasal lavage fluid of subjects with allergic rhinitis after allergen challenge. Following nasal challenge with ragweed pollen, leukotrienes were recovered from nasal lavage fluid from subjects with clinical rhinitis due to ragweed, but were not detectable in nasal lavage fluid from normal subjects (Creticos *et al.*, 1984). It is possible that the main source of LTB₄ and LTC₄, in the early reaction, is mast cells, which have been shown to release LTB₄ and LTC₄ upon the coupling of mast cell-bound IgE to a specific allergen (Pipkorn *et al.*, 1987). Recovery of all three cysteinyl leukotrienes in the lavage fluid, although LTC₄ predominates, in both the early and late phase response (Pipkorn *et al.*, 1987), demonstrated that the sequential bioconversion of LTC₄ can occur in the nose.

In an *in vitro* study, LTC₄ and LTD₄ caused human airway mucus secretion (Marom *et al.*, 1982). Nasal provocation with LTC₄ and LTD₄ has been reported to increase nasal airway resistance but not the amount of nasal secretions, itching and sneezing (Bisgaard *et al.*, 1986; Miadonna *et al.*, 1987). Topical nasal application of LTD₄ in normal volunteers has the capacity to induce a dose-related increase in nasal blood flow, as measured by laser-Doppler flow-sensing devices (Bisgaard *et al.*, 1986). The changes are associated with an increase in nasal airway resistance. LTD₄-induced an

increase in nasal airway resistance which exceeds that induced by histamine, but is quite similar to that achieved by antigen inhalation (Okuda *et al.*, 1988).

A 5-lipoxygenase inhibitor, A-64077, which inhibits the formation of LTB₄ as well as LTD₄ and other cysteinyl leukotrienes, has some benefit in allergen challenge of patients with allergic rhinitis. A-64077 reduced nasal congestion, as measured by symptoms scores, following allergen challenge (Knapp *et al.*, 1990). Another separate investigation on the effect of another 5-lipoxygenase inhibitor, A-78773, after allergen challenge, has quantitatively confirmed the beneficial role of 5-lipoxygenase inhibitors in reducing the increase in nasal airway resistance measured by rhinomanometry (Howarth & Harrison, 1995). These studies demonstrated that there is a possibility of leukotriene involvement in nasal allergic rhinitis and that the inhibition of leukotriene synthesis may provide a beneficial treatment in allergic rhinitis.

1.5.3 PLATELET-ACTIVATING FACTOR (PAF)

1.5.3.a Synthesis and catabolism of PAF

Platelet-activating factor (PAF) is a highly potent inflammatory mediator. Like prostaglandins and leukotrienes, it is formed by the action of phospholipase A₂ (PLA₂) on membrane phospholipids. It has been referred to by other names such as acetyl-glycerol-ether-phosphorylcholine (AGEPC) and PAF-acether but the original nomenclature, PAF, will be used in this context. The biosynthesis of PAF occurs as a two-step process in which a PLA₂ enzyme hydrolyses acyl-PAF to lyso-PAF. This lyso-PAF which is both a precursor and metabolite of PAF is then acetylated to PAF by an acetyl transferase enzyme that is present in a variety of cells. PAF in turn can be deacetylated to lyso-PAF by the action of an acetyl hydrolase enzyme that is widely distributed and has high activity (figure 1.5). Acetyl hydrolase is extremely active so that after an intravenous injection of PAF, 70% of the PAF is present as lyso-PAF within 1 minute (Lartigue-Mattei *et al.*, 1984).

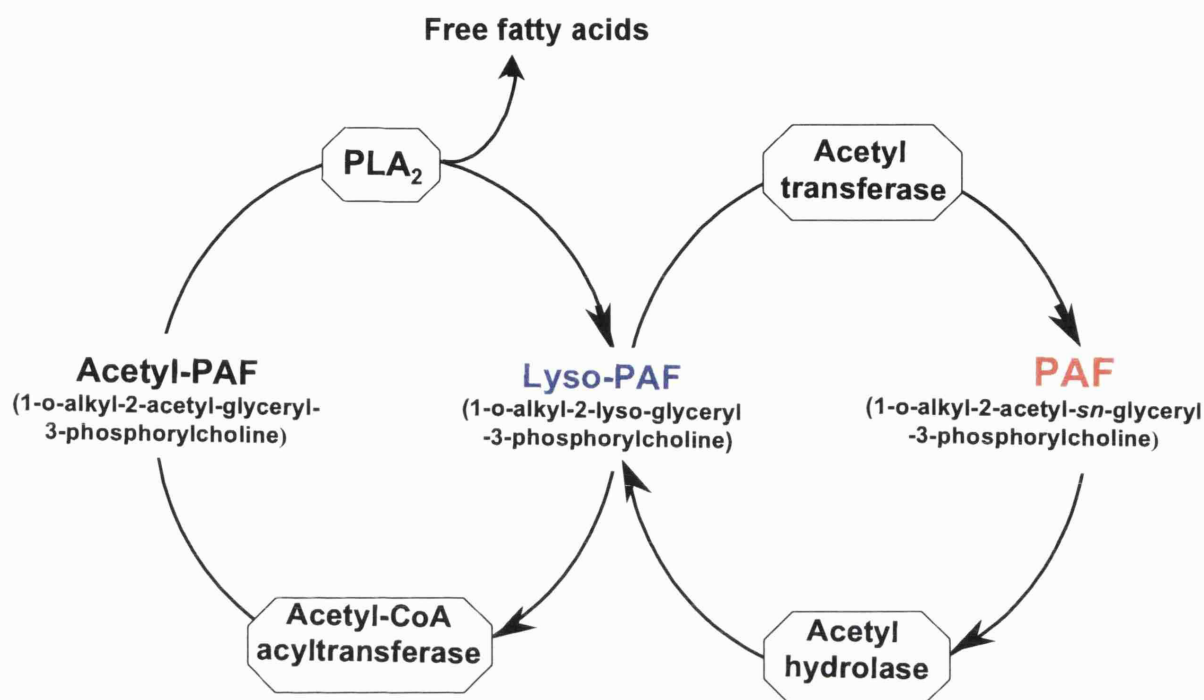


Figure 1.5: Synthesis and metabolism of PAF (Barnes *et al.*, 1988; Snyder *et al.*, 1990; Page *et al.*, 1994).

There is an alternative route for the formation of PAF, detected in rat kidney (Renooij & Snyder, 1981; Pirotzky *et al.*, 1984), which involves the direct transfer of 1-o-alkyl-2-acetyl-glycerol to PAF by the enzyme phosphocholine transferase. This pathway is known as the *de novo* pathway. Inflammatory cells synthesise PAF through the two-step pathway involving the rate limiting enzyme, acetyl transferase. The *de novo* pathway, on the other hand, is utilised in non-inflammatory cells and is believed to provide endogenous PAF for physiological functions (Snyder *et al.*, 1990). The two-step pathway therefore plays a role in inflammation and allergic reactions.

Numerous types of cells have been reported to synthesize and release PAF in response to various stimuli. These cells include neutrophils (Camussi *et al.*, 1981; Lee *et al.*, 1982; Clay *et al.*, 1984), platelets (Chignard *et al.*, 1980), macrophages (Arnoux *et al.*, 1981 and 1982), eosinophils isolated from patients with eosinophilia (Lee *et al.*,

1984) and mast cells (Schleimer *et al.*, 1986). Endothelial cells also produce PAF following stimulation with thrombin, interleukin 1 (IL-1) or tumor necrosis factor (TNF) (Camussi *et al.*, 1983; Prescott *et al.*, 1984; Zimmerman *et al.*, 1990). The production of PAF by endothelium was thought to play a role in the adhesion of circulating cells, such as neutrophils and eosinophils (Zimmerman *et al.*, 1990).

1.5.3.b Receptors and the biological action of PAF

PAF is a potent bronchoconstrictor in several species, including man (Cuss *et al.*, 1986). Inhalation of PAF produced a significant and long-lasting increase in bronchial responsiveness in normal volunteers (Cuss *et al.*, 1986; Kaye & Smith, 1990) as well as volunteers with asthma (Cuss *et al.*, 1986). The intradermal injection of PAF produces a biphasic cutaneous response characterised by an immediate wheal and flare and a delayed (3 to 6 hours) reaction characterised by erythema and hyperalgesia (Basran *et al.*, 1984). PAF induces microvascular leakage in several tissues at concentrations approximately 1000 times less than that of histamine (Morley *et al.*, 1983; Evan *et al.*, 1987), and it increases airway secretions and epithelial permeability (Rogers *et al.*, 1990a). The intravenous administration of PAF rapidly induces hypotension, due to increased capillary permeability with fluid loss and depressed myocardial contractility (Damas *et al.*, 1989).

One of the most prominent properties of PAF is its ability to activate a wide range of inflammatory cells. In addition to being able to activate platelets, the first cell type shown to respond to PAF (Chignard *et al.*, 1979), PAF target cells include eosinophils, macrophages, neutrophils, endothelial cells, T- and B-lymphocytes and neurones (Colditz & Topper, 1991; Huang *et al.*, 1996). PAF induces the aggregation of inflammatory cells and the release of other inflammatory mediators. PAF also induces free radical production and leukotriene synthesis in neutrophils and eosinophils (O'Flaherty & Wykle, 1983). The intradermal injection of PAF, in human skin, induced neutrophil infiltration (Archer *et al.*, 1985). In contrast, in atopic volunteers, PAF results in a selective eosinophil infiltration when injected intradermally (Henocq & Vargaftig, 1986). Atopic individuals may, therefore,

respond differently to PAF in comparison with normal individuals. Eosinophil responsiveness is quantitatively different when these cells originate from atopic subjects, and PAF may enhance their inflammatory response. In other words, there is a close relationship between PAF and eosinophils. Eosinophils are the cells most sensitive to activation by PAF. PAF is one of the most potent eosinophil chemotactic factors (Wardlaw *et al.*, 1986). When the potency of PAF was compared to histamine and LTB₄, the ability of histamine and LTB₄ to cause accumulation of eosinophils was minimal in comparison to PAF (Tamura *et al.*, 1987). Since eosinophils themselves are a rich source of PAF (Chung & Barnes, 1992), they can attract further eosinophils, thus there is potential for a continued inflammatory reaction. Furthermore, PAF is very effective in stimulating human eosinophils to release basic proteins (Capron *et al.*, 1989; Kroegel *et al.*, 1988), and in increasing expression of IgE-receptors on eosinophils (Moqbel *et al.*, 1990).

Specific receptors for PAF have been identified on its target cells such as platelets (Valone *et al.*, 1982), eosinophils (Kroegel *et al.*, 1989) and neutrophils (Valone & Goetzl, 1983; O'Flaherty *et al.*, 1986), by the use of [³H]PAF as a radioligand. Specific PAF antagonists exhibit different potencies in human platelets and neutrophils (Hwang *et al.*, 1990). Based on this observation the presence of PAF receptor subtypes has been suggested. In addition there appear to be two receptor types for PAF on eosinophils; one causing EPO release at low concentrations of PAF and the other causing O₂ release at high concentrations of PAF (Kroegel *et al.*, 1989).

1.5.3.c PAF and rhinitis

PAF and its metabolite (lyso-PAF) were detected in nasal secretion after antigen challenge in patients with seasonal rhinitis (Meslier *et al.*, 1988; Miadonna *et al.*, 1989; Shin *et al.*, 1994) although PAF was detected only in small amounts. Intranasal challenge with PAF at doses of 157 µg or 314 µg induced an increase in nasal airway resistance and symptom scores, but no differences were observed between normal and rhinitic subjects. Lower doses of PAF did not induce nasal obstruction (Pipkorn *et al.*, 1984). Interestingly, much lower doses of PAF (30 µg and 60 µg) induced an increase

in responsiveness to inhaled histamine and bradykinin (Austin & Foreman, 1993; Turner *et al.*, 2000). In addition, PAF pretreatment induced an increase in responsiveness to allergen challenge (Anderson & Pipkorn, 1988). Neither nasal symptoms nor changes in nasal airway resistance were observed after nasal challenge with lyso-PAF (Leggieri *et al.*, 1991). Lyso-PAF also failed to induce hyperresponsiveness to histamine or bradykinin (Austin & Foreman, 1993; Turner *et al.*, 2000). The evidence suggests that PAF is a mediator of nasal hyperresponsiveness.

The mechanism involved in PAF-induced hyperresponsiveness may be the result of its ability to recruit inflammatory cells and induce the release of free radicals and proteins from these cells. Intranasal challenge with PAF led to an increase in both neutrophil (Miadonna *et al.*, 1996) and eosinophil (Klementsson & Anderson, 1992; Tedeschi *et al.*, 1994a and 1994b) numbers, which appear earlier in the allergic rhinitic, compared to normal subjects. The increase in the number of neutrophils and eosinophils is accompanied by the release of MPO (Miadonna *et al.*, 1996) and ECP respectively (Tedeschi *et al.*, 1994a; Austin & Foreman, 1993). Austin and Foreman also provided evidence that free radical production induced by PAF may contribute to the hyperresponsiveness and the activation of eosinophils. Pretreatment with vitamin E, an oxygen free radical scavenger, caused an attenuation of PAF-induced nasal hyperresponsiveness and a reduction in the ECP levels in nasal lavage fluid.

1.5.4 KININS

Kinins are involved in physiological regulation of all major systems in the body and are mediators of a wide range of pathologies, including inflammation. Exogenous kinins have been shown to produce the classical signs of inflammation. The extent of kinin involvement in pathologies has begun to be appreciated, kinin antagonists have been shown to increase survival rate in rat and rabbit models of endotoxin shock (Whalley *et al.*, 1992).

1.5.4.a Synthesis and catabolism of kinins.

Kinins are vasoactive peptides that are formed by the action of the enzyme kallikrein on precursor kininogens (α_2 -globulin). The major kinins are bradykinin (BK) and kallidin (KD) or lysyl-BK. Kininogens are found in both high-molecular-weight (HMWK) and low-molecular-weight (LMWK) forms. Both kininogens consist of three domains; an amino-terminal heavy chain, the kinin moiety and a carboxyl-terminal light chain, figure 1.6.

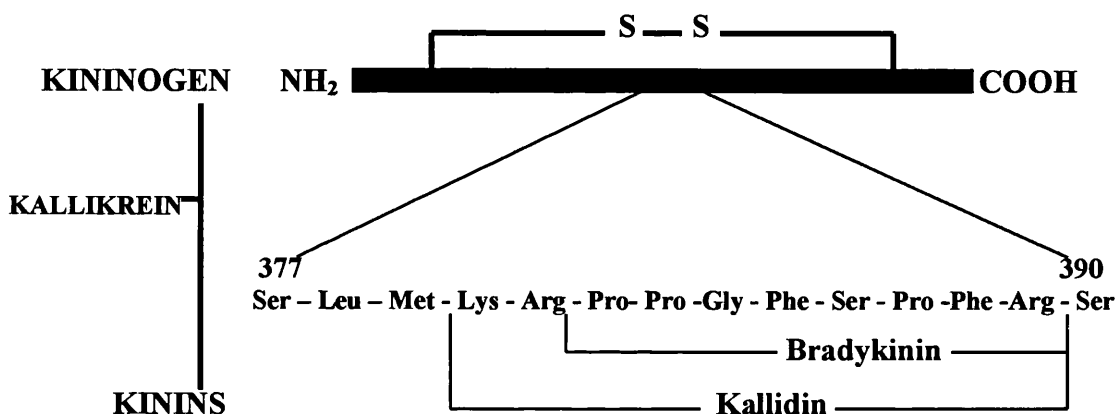


Figure 1.6: A partial amino acid sequence for kininogen and its cleavage sites by kallikreins, resulting in the generation of bradykinin and kallidin (Regoli *et al.*, 1980; Zuraw *et al.*, 1994).

Two primary kallikrein enzymes that cleave kininogens are tissue kallikrein and plasma kallikrein. Tissue kallikrein, is also known as glandular kallikrein, releases kallidin from both HMWK and LMWK. It does not circulate in blood but is found to be expressed in many mammalian organs, including salivary glands, pancreas, kidney, nose and lung. HMWK is the exclusive kininogen substrate for plasma kallikrein. In plasma, the enzyme is present in an inactive form, pre-kallikrein, which is generated in the liver. Plasma pre-kallikrein is activated by the Hageman factor which is activated when it comes into contact with negatively charged surfaces. HMWK circulates in a complex with plasma pre-kallikrein and is ready to release bradykinin at the site where contact with the negatively charged surfaces promotes the interaction

of pre-kallikrein and Hageman factor and thus activation of kallikrein and the generation of bradykinin.

Bradykinin is inactivated by enzymes called kininases. The main kininases are carboxypeptidase N (CPN) also known as kininase I, angiotensin-converting enzyme (ACE) or kininase II, neutral endopeptidase (NEP) and aminopeptidases. The positions where the kininases cleave bradykinin and kallidin are shown in figure 1.7.

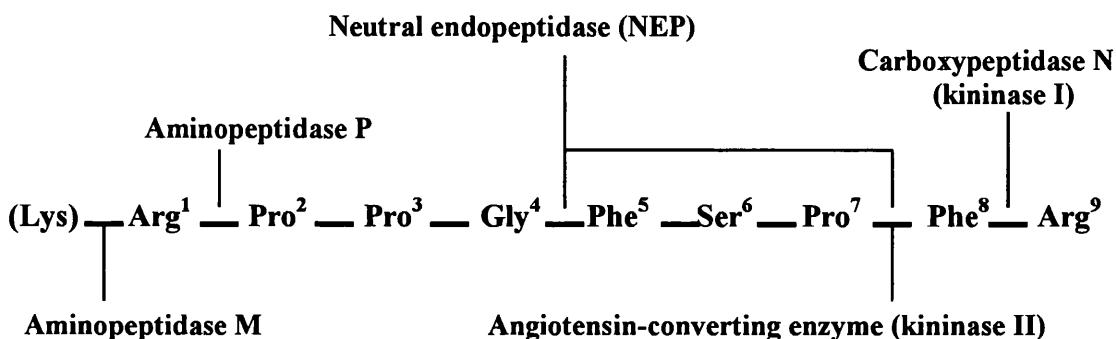


Figure 1.7: Kininase degradation of kinin. The numbering of the amino acids in the displayed peptide refers to bradykinin. The additional Lys amino acid of kallidin is shown in brackets (Zuraw *et al.*, 1994).

The precise metabolic pathway of kinin degradation depends on the site of kinin generation. In plasma, kinins are principally inactivated by kininase I to des-Arg⁹-BK and des-Arg¹⁰-KD which are active metabolites of the kinins. These metabolites are the primary agonists for B1-receptors, one of the two main classes of kinin receptors. ACE or kininase II is formed on the plasma membrane of vascular endothelial cells. It is widely distributed in a variety of vascular beds; in pituitary gland, brain, kidney and lung. ACE is very effective in kinin inactivation as it eliminates the biological activities of both bradykinin and kallidin completely, by removing the C-terminal dipeptide Phe⁸-Arg⁹. NEP, like ACE, is found in high concentrations in tissues especially in kidney and lung. It is also found in human neutrophils (Skidgel *et al.*,

1991a). In addition to cleaving kinins, NEP can effectively inactivate substance P, but not neurokinin A. Kallidin is rapidly converted in plasma to bradykinin by aminopeptidase M. Bradykinin can then be cleaved by aminopeptidase P.

Kinins exist in circulating blood in very low concentrations as the enzymes responsible for their degradation are multiple, very efficient and distributed in blood and in tissues. The half-life of kinins is, therefore, very short.

1.5.4.b Receptors and the biological action of kinins

The actions of kinins are mediated through at least two different types of receptor designated as B₁ and B₂. The two kinin receptors are defined by the rank potency of their agonists and the action of selective antagonists. Tissues that respond to des-Arg⁹-BK and des-Arg¹⁰-kallidin more sensitively than to bradykinin and kallidin are considered to have B₁ receptors while tissues responding to bradykinin and kallidin more sensitively than des-Arg⁹-BK and des-Arg¹⁰-kallidin are considered to have B₂ receptors. The B₁ kinin receptor is expressed in only a few tissues but appears to be up-regulated in some forms of tissue injury and inflammation (Marceau *et al.*, 1995). B₂ kinin receptors mediate most of the *in vivo* effect of kinins such as increased vascular permeability, vasodilatation, induction of pain, bronchoconstriction and regulation of cardiovascular system (Regoli & Barabe, 1988a and 1988b; Regoli & Barabe, 1980). Bradykinin is spasmogenic for several types of smooth muscle including that of the intestine and the uterus. Bradykinin is a potent bronchoconstrictor in both asthmatic and non-asthmatic tissue *in vitro* and *in vivo* (Fuller *et al.*, 1987b; Polosa & Holgate, 1990). Bradykinin is also known to stimulate ion transport and fluid secretion by various epithelia, including those of the intestine and the airways.

Bradykinin can produce either dilation or constriction of blood vessels. However dilation occurs in the majority of the vascular beds. Bradykinin exerts its constrictor effect on veins, presumably by the stimulation of the venous smooth muscles or by

the release of the vasoconstrictor prostaglandin $\text{PGF}_{2\alpha}$ (Chaud *et al.*, 1997). The effects of bradykinin and kallidin are often part of a complex cascade of events which include other mediators. The vasodilator action of kinins is partly due to the fact that kinins stimulate the generation of prostacyclin PGI_2 (Ichinose *et al.*, 1990) and the release of nitric oxide (NO). Bradykinin has been shown to increase the production of prostaglandins *in vivo* (Vane *et al.*, 1976) and *in vitro* (Terragno & Terragno, 1979). Among the vasoactive peptides tested as prostaglandin releasers, bradykinin appears to be the most active (Regoli *et al.*, 1977).

The vasodilatation evoked by bradykinin has been shown to be dependent on the presence of an intact endothelium, which, in turn, produces a substance that acts on the vascular smooth muscle to cause relaxation (Furchgott & Zawadzki, 1980). This substance was designated as 'endothelium-derived relaxing factor' (EDRF) and later identified as nitric oxide (NO). NO has now been recognised as a mediator of several biological actions. It is a neurotransmitter and neuromodulator in the central nervous system (Garthwaite *et al.*, 1991). NO is produced from the reaction between molecular oxygen (O_2) and L-arginine by a reaction catalysed by NO synthase. In addition to acting on endothelial cells to release prostaglandins and NO, kinins cause contraction of endothelial cells which opens spaces in the capillary wall. This increased vascular permeability, results in extravasation of plasma and protein into the extravascular compartments, contributing to oedema formation.

Kinins are prominent mediators of pain, stimulating all pain-fibre classes (Carratu *et al.*, 1989). They play a major role in the initiation and maintenance of the inflammatory pain process. Bradykinin receptors are found on sensory neurones (Steranka *et al.*, 1988). Furthermore bradykinin has been shown to induce neuronal discharges in sensory C fibres *in vitro* and *in vivo* (Dray *et al.*, 1992), and to induce nociceptive responses indicative of pain in animals (Khasar *et al.*, 1993), and overt pain in man (Whalley *et al.*, 1987; Kingden- Milles *et al.*, 1992). In addition to stimulating sensory afferents, bradykinin causes the release of neuropeptides from sensory nerves (Geppetti *et al.*, 1988; Geppetti *et al.*, 1993; Saria *et al.*, 1988).

Kinins can activate inflammatory cells and amplify the release or production of other putative mediators (Proud *et al.*, 1988). IL-1 and BK act synergistically to stimulate prostaglandin synthesis in human synoviocytes and fibroblasts (Lerner & Modeer, 1991). Kinins have also been shown to induce mediator release from rodent mast cells (Johnson & Erdos, 1973; Ishizaka *et al.*, 1985).

Kinins have been shown to reproduce the basic symptoms of inflammation in several animal species (Miles & Wilhelm, 1960). Activation of kallikrein occurs in inflamed tissues, and the degradation of kinins may be reduced by the decreased pH of oedematous fluid, thus prolonging inflammation (Garcia *et al.*, 1978). The ability of kinins to interact with other inflammatory systems and neurones make them potential mediators of inflammation.

1.5.4.c Bradykinin as a mediator of rhinitis

A large number of studies have been carried out, in the last few years, on the relevance of kinins in allergic rhinitis. Many studies have demonstrated the presence of kinins in nasal secretion during allergic reactions. Kinins are detected in nasal secretions of patients suffering from seasonal allergic rhinitis as a result of natural allergen exposure (Svensson *et al.*, 1990) and in nasal secretion following experimental allergen challenge in rhinitic subjects (Naclerio *et al.*, 1985a; Proud *et al.*, 1983 and 1989). This increase in the kinin level has been shown in both the immediate allergic response and the late phase response (Naclerio *et al.*, 1985a; Baumgarten *et al.*, 1985; Proud *et al.*, 1989). Other challenges such as cold dry air (Togias *et al.*, 1985a) as well as experimental rhinovirus infections of the nose have led to increased levels of kinins in nasal lavage (Naclerio *et al.*, 1988b). The nasal fluid obtained from the patients suffering from natural rhinovirus infection contains high amounts of kinins and albumin (Proud *et al.*, 1990). Kinins, cytokines and chemokine levels are increased above baseline in nasal secretions during induced rhinovirus infection, whereas histamine levels remained constant (Naclerio *et al.*, 1988b; van Kempen *et al.*, 1999; Gern *et al.*, 2000).

The kinins detected are a mixture of bradykinin and kallidin, with the bradykinin concentration greater than that of kallidin. The presence of both bradykinin and kallidin suggests the presence of multiple kallikrein activities. Nasal allergen challenge has been shown to result in the entry of both HMWK and LMWK into nasal secretion (Baumgarten *et al.*, 1985 and 1986a). Upon entering, HMWK and LMWK can then be substrates for plasma and glandular kallikrein. Glandular kallikrein has been demonstrated to increase during experimentally induced allergic rhinitis (Baumgarten *et al.*, 1986c) which contributes to the formation of kallidin. Increased levels of plasma pre-kallikrein were also detected. Gel filtration showed that plasma pre-kallikrein is activated during the allergic response and contributes to the formation of bradykinin (Baumgarten *et al.*, 1986b). Entry of HMWK, LMWK and plasma pre-kallikrein into the nasal secretion was presumably due to increased transudation following the immediate response to allergen as suggested by the increase in albumin (Baumgarten *et al.*, 1985 and 1986a). Pre-kallikrein may be activated in nasal secretions by mast cells which have been reported to contain a pre-kallikrein activator (Newball *et al.*, 1981). In addition, the negative surface of the nasal mucosa may allow the activation of Hageman factor (Kaliner *et al.*, 1984) with subsequent conversion of pre-kallikrein to kallikrein. During the allergic response, nasal secretion contains aminopeptidase and carboxypeptidase activities in addition to low levels of ACE (Proud *et al.*, 1987). Aminopeptidase converts kallidin to bradykinin while carboxypeptidase converts bradykinin and kallidin to des-Arg⁹-BK and des-Arg¹⁰-KD.

Nasal challenge with bradykinin leads to an induction of rhinitic symptoms and increased vascular permeability (Churchill *et al.*, 1991; Proud *et al.*, 1988). Bradykinin challenges induced an increase in albumin and TAME-esterase activity in nasal fluid and caused sore throats (Proud *et al.*, 1988; Doyle *et al.*, 1990) in both atopic and non-atopic subjects. Furthermore, albumin levels induced by bradykinin challenge were significantly higher in subjects with seasonal allergic rhinitis, challenged out of season, than in normal subjects (Brunner *et al.*, 1991). Nasal challenge with kallidin also induces symptoms of rhinitis (Rajakulasingham *et al.*, 1991). Both kallidin and bradykinin have been widely shown to induce a dose-

dependent increase in nasal airway resistance which was associated with nasal discomfort in both normal and rhinitic subjects (Doyle *et al.*, 1990; Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a and 1994b). Des-Arg⁹-BK and des-Arg¹⁰-KD, B₁ agonists, have no effect on nasal airway resistance, albumin release or symptoms scores (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a; Churchill *et al.*, 1991). These findings indicated that the nasal effects of kinins are mediated through the B₂ receptor subtype. Bradykinin-induced symptoms and albumin release were inhibited in a dose-related manner by pre-administration of Hoe-140, a B₂ receptor antagonist (Proud *et al.*, 1995). In addition, pretreatment with Hoe-140 produced an inhibition of increased nasal airway resistance and release of albumin induced by bradykinin in normal subjects and by house dust mite antigen in subjects with allergic rhinitis (Austin *et al.*, 1994c). Furthermore, it was demonstrated by Dear (1996b) that bradykinin and B₂ receptor antagonists, including Hoe 140, were able to displace ¹²⁵I-Hoe 140 from binding sites in membranes from human inferior turbinates whereas des-Arg⁹-BK and the B₁ receptor antagonist, des-Arg⁹-Hoe 140, failed to displace the ¹²⁵I-Hoe 140 from binding sites. These findings further confirm the presence of B₂ receptor subtypes in the nasal airway.

Autoradiographic distribution of ¹²⁵I-BK binding sites in human inferior turbinate revealed ¹²⁵I-BK binding sites on the walls of small arteries, arterioles, capillaries, small venules and venous sinusoids, and on submucosa nerve fibres, but no specific binding to submucosal glands or epithelium (Baraniuk *et al.*, 1990e). The binding of bradykinin to vessels is consistent with an effect of bradykinin in inducing an increase in nasal airway resistance and an increase in vascular permeability. Kinins produce arteriolar dilation which causes an increase in pressure and flow in the capillary bed thus favouring efflux of fluid from blood to tissues. This effect may be facilitated by increased capillary permeability as a result of the formation of large pores or gaps in the capillary endothelium, which is a consequence of kinin action on endothelial cells to produce cell contraction, and by increased venous pressure secondary to the constriction of veins. Plasma extravasation (indicated by an increased level of albumin) and oedema (nasal blockage) are, therefore, results of these changes. The

formation of nitric oxide also contributes to an increase in plasma extravasation and in nasal airway resistance (Dear *et al.*, 1996a).

Bradykinin at high concentrations (1000 nM) has been shown to stimulate contralateral secretion in subjects with severe perennial allergic rhinitis but not in normal subjects (Baraniuk *et al.*, 1994b). This observation suggests an increase in nasal responsiveness to bradykinin in chronic perennial rhinitic subjects. This bradykinin-induced reflex-mediated glandular secretion can be inhibited by pretreatment with ipratropium bromide (Baraniuk *et al.*, 1994a), indicating cholinergic reflexes.

1.5.5 CYTOKINES AND CHEMOKINES

1.5.5.a Cytokines

Cytokines are also important mediators of allergic rhinitis and they are involved in cell-to-cell communication. They are produced by, and act on, many different cells such as T cells, macrophages and mast cells. They may influence other cells to produce other cytokines leading to a cascade of events (Hamblin *et al.*, 1994). Allergic rhinitis is thought to result from a series of cellular interactions. Stimulation of T-helper cells releases cytokines such as IL-4, important for IgE synthesis (Hamblin *et al.*, 1994; Forsythe & Ennis, 1998), and IL-5, which is important for eosinophil differentiation and activation (Sehmi *et al.*, 1992; Bates *et al.*, 2000; Foster *et al.*, 2001; Lampinen *et al.*, 2001). The synthesis and the release of IgE from B cells results in mast cell degranulation when antigen binds to IgE on the mast cell membrane (Benson *et al.*, 2001). Mast cells also produce and release a number of cytokines including TNF α , IL-4, IL-5, IL-6 and IL-8 (Forsythe & Ennis, 1998; Wilson *et al.*, 2000), each of which can have an effect on other cells. For example IL-8 and IL-5 cause the influx of eosinophils and neutrophils in to the nose (Kramer *et al.*, 2000; Benson *et al.*, 1999 and 2001). Local release of cytokines in the nasal mucosa from stimulated cells can, therefore, produce a wide range of effects.

However, lack of suitable antagonists for cytokines makes establishing the exact role of each cytokine in allergic rhinitis a difficult task.

Several studies have reported that cytokines are released into nasal secretion. Elevated levels of IL-5 were detected in nasal secretion from seasonal allergic rhinitis patients compared with controls (Kramer *et al.*, 2000). Elevated levels of IL-1 β , TNF α , IL-6 and IL-8 have been shown to be present in nasal secretions from allergic rhinitis patients under experimental and natural conditions (Bachert *et al.*, 1995). The concentration of IL-6, IL-8 and GM-CSF has been shown to increase in the nose after antigen challenge in house dust mite allergic rhinitis (Ohkubo *et al.*, 1998). IL-1 β and TNF α act on endothelial cells to induce prostacyclin synthesis, expression of adhesion molecules and synthesis of cytokines (Hamblin *et al.*, 1994). IL-6 enhances expression of receptors for the T cell growth factor and IL-2, and it induces B cell differentiation (Morgan *et al.*, 1990; Splawski *et al.*, 1990). Cytokines therefore may play an important role in the pathogenesis of the allergic reaction, or its modulation.

1.5.5.b Chemokines

A family of chemotactic cytokines known as chemokines comprises low-molecular-weight proteins that regulate the complex and precise recruitment of immune cells into inflammatory sites. They are secondary pro-inflammatory mediators that are induced by primary pro-inflammatory mediators such as tumor necrosis factor (TNF) (Graves *et al.*, 1995; Steube *et al.*, 2000; Sebastiani *et al.*, 2002) and interferon (IFN) (Chuluyan *et al.*, 1998; Neumann *et al.*, 1998; Fantuzzi *et al.*, 2001). Chemokines can be divided, according to structural variations in the position of cysteine residues, into three subfamilies, alpha-chemokines or CXC chemokines, beta-chemokines or CC chemokines, and gamma-chemokines or C chemokines (Graves *et al.*, 1995). CXC chemokines are a group of chemokines such as IL-8 and neutrophil-activating peptide-2 (NAP-2), which are strong neutrophil attractants with no effect on monocytes (Proost *et al.*, 1996; Balkwill *et al.*, 1998; Mukaida *et al.*, 1998; Fujiwara *et al.*, 2002). CC chemokines, including monocyte chemotactic protein (MCP)-1, MCP-2, MCP-3 and MCP-4, monocyte inflammatory proteins (MIP)-1 α and MIP-1 β ,

RANTES (regulated on activation, normal T expressed and secreted) and eotaxin, are a group of chemokines that are chemoattractants for lymphocytes, monocytes, eosinophils and basophils but not neutrophils (Weber *et al.*, 1995; Balkwill *et al.*, 1998; Mukaida *et al.*, 1998; Conti *et al.*, 1997 and 1999). C chemokines are a group of chemokines that are chemoattractants for lymphocytes only (Proost *et al.*, 1996; Balkwill *et al.*, 1998; Mukaida *et al.*, 1998; Moser *et al.*, 2001).

Chemokines IL-8, RANTES, MIP-1alpha, and MCP-1 have been shown to increase in nasal secretions obtained from subjects with a virus infection of the nasal airway (Bonville *et al.*, 1999; Teran *et al.*, 1999; Noah *et al.*, 2000). Increased levels of IL-8, RANTES and MIP-1alpha were observed in the nasal secretions of allergic rhinitis following antigen challenge (Weido *et al.*, 1996). Increased expression of MCP-3 and MCP-4 was observed after antigen challenge (Christodouloupoulos *et al.*, 1999). MCP-1 has been shown to provoke mast cell aggregation and [³H]5HT-release from cultured mast cells (Conti *et al.*, 1995). Histamine has been shown to induce CC chemokine production in the nasal mucosa of patients with allergic rhinitis (Fujikura *et al.*, 2001). In addition to its chemotactic activity, MCP-4 also stimulates histamine release from basophils (Garcia-Zepeda *et al.*, 1996). This indicates that there may be a prolonged inflammatory cycle in the histamine-MCP axis in allergic rhinitis. mRNA expression for eotaxin has been shown to be increased in nasal polyps with eosinophilia (Jahnsen *et al.*, 1999; Shin *et al.*, 2000). Nasal provocation with eotaxin has been shown to induce an influx of eosinophils into the nasal mucosa (Gorski *et al.*, 2002). Expression of eotaxin in the nasal mucosa of grass-pollen allergic rhinitis patients has been shown to be upregulated during the pollen season (Pullerits *et al.*, 2000). Thus, eotaxin with its preferential action on eosinophils, is likely to play an important role in allergic rhinitis. Chemokines, therefore, may contribute significantly in the pathogenesis of allergic rhinitis.

1.6 NERVES AND NEUROTRANSMITTERS

In nasal mucosa, neuronal control plays a major role in the regulation of the glandular and vascular processes that generate nasal mucus and govern nasal airflow. Three types of nerves: parasympathetic, sympathetic and sensory, innervate the nasal mucosa, figure 1.8. These nerves release a specific combination of transmitters which combine to regulate these processes. The balance between the effects of parasympathetic and sympathetic activities may regulate nasal homeostasis, while the sensory system detects the conditions of inspired air and responds to inhaled noxious agents and mucosal injury.

1.6.1 Sympathetic Nerves

Sympathetic nerves contain noradrenaline or noradrenaline plus neuropeptide Y (NPY): both are potent vasoconstrictors (Uddman & Sundler, 1986). Noradrenergic nerve fibres extensively innervate arterial vessels and arteriovenous anastomoses, but are only rarely found in glands (Uddman & Sundler, 1986; Klaassen *et al.*, 1988). Activation of the nerve results in vasoconstriction (Uddman & Sundler, 1986). These nerves mediate the increase in nasal patency that occurs during exercise (Richerson & Seeborn, 1968). Noradrenaline acts upon α - and β -adrenoreceptors. β -adrenergic agonists are mild vasodilators and have no effect on glandular or vascular secretory processes in human nasal mucosa *in vivo*, or glandular secretion *in vitro* (Mullol *et al.*, 1992b). Noradrenaline acts upon α_1 - and α_2 -adrenoreceptors to induce vasoconstriction (Berridge & Roach, 1986). α_1 -adrenergic agonists induce only a limited amount of serous cell and mucous cell secretion from human nasal mucosa *in vitro* and *in vivo* (Mullol *et al.*, 1992b). Agonists of α_1 - and α_2 -adrenoreceptors are popular nasal decongestants as they effectively reduce mucosal thickness *in vivo*. Allergic and control nasal mucosal tissues contain identical numbers of α_1 - and α_2 -adrenoreceptors, indicating that modulation of adrenergic receptor systems does not occur in allergic rhinitis (van-Megen *et al.*, 1991).

In some populations of sympathetic neurons, NPY is present with noradrenaline. This peptide has many of the same actions as noradrenaline. NPY-induced vasoconstriction is slower in onset but longer in duration, than that of noradrenaline. The locations of NPY nerve fibres and NPY binding sites are on arterioles and arteriovenous anastomoses (Uddman & Sundler, 1986; Baraniuk *et al.*, 1990a). Exogenous administration of NPY to human nasal mucosa has been shown to reduce nasal airflow resistance and albumin exudation without affecting submucosal gland secretion (Baraniuk *et al.*, 1992c).

Nasal airflow is normally asymmetrical, and subject to spontaneous reciprocal changes which are often referred to as 'the nasal cycle'. The nose receives both sympathetic and parasympathetic innervation and in normal nasal cycles there is an alternating dominance of sympathetic activity on one side with concurrent parasympathetic dominance on the other.

1.6.2 Parasympathetic Nerves

Parasympathetic nerves originate in the seventh cranial nerve, synapse in the sphenopalatine ganglion and pass through the vidian nerve. Parasympathetic discharge will release acetylcholine (Ach), vasoactive intestinal polypeptide (VIP) and the closely related peptide, peptide histidine methionine (PHM). The parasympathetic nervous system regulates glandular and vasomotor processes in the nasal mucosa (Konno & Togawa, 1979; Baraniuk *et al.*, 1991a). Nasal mucosa nerve fibres that contain acetylcholinesterase (AChE), a marker of cholinergic nerves, are densely distributed around submucosal glands, at the basement membrane and within the epithelium, with some nerves in close contact with goblet cells. They also innervate arterial vessels and sinusoids (Ishii & Toriyama, 1972; Katahashi *et al.*, 1997).

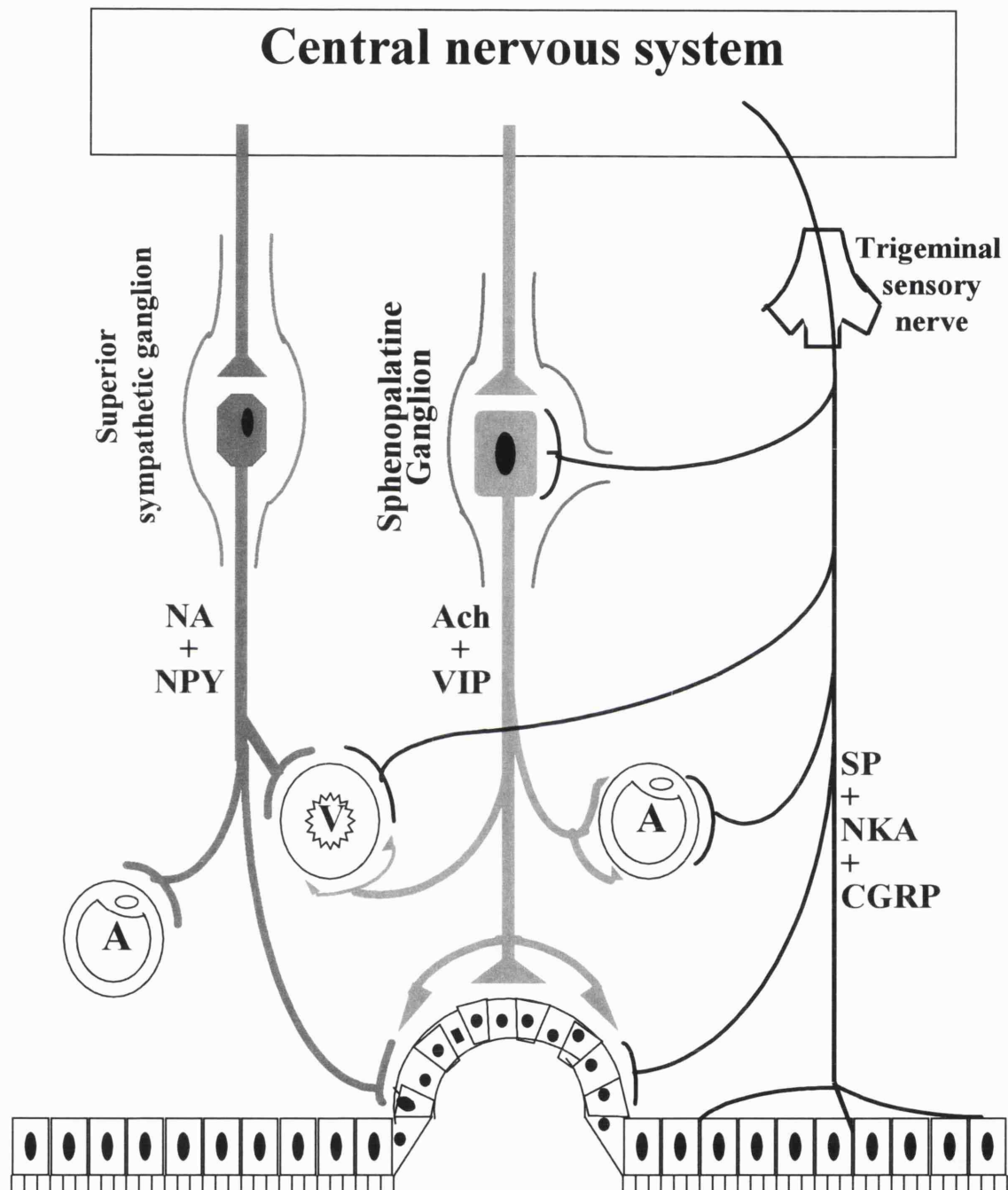


Figure 1.8: Schematic illustration of nerve innervation of the nasal mucosa, (A) represents arteries and (V) represents venous sinusoids (Uddman *et al.*, 1986; Baraniuk *et al.*, 1995).

Acetylcholine acts upon peripheral muscarinic receptors. Five muscarinic receptor genes (M_1 to M_5) have been cloned (Barnes *et al.*, 1989), but only agonists and antagonists to M_1 , M_2 and M_3 subtypes are available. The location of Ach-binding sites in human nasal mucosa has been identified by the use of autoradiography, radiolabelled ligand binding, competitive binding analysis and in situ hybridization. M_1 binding sites are present on the epithelium and glands, whereas M_3 binding sites are present on the epithelium, submucosal glands and vessels. M_2 binding sites have not been found in nasal mucosa (Mak & Barnes 1990; Baraniuk *et al.*, 1992a; Okayama *et al.*, 1993). The distribution of muscarinic receptors on glands and the epithelium is consistent with the well-recognised potency of muscarinic agonists as secretagogues (Baroody *et al.*, 1996; Mygind & Dahl, 1996). Stimulation of parasympathetic nerves leads to glandular secretion, which can be reduced, or eliminated, by the non-selective muscarinic antagonists, atropine and ipratropium bromide (Konno & Togawa, 1979; Raphael *et al.*, 1988 and 1989a; Baroody *et al.*, 1992). *In vitro*, methacholine (analogue of Ach) has been shown to stimulate glandular secretion (Patow *et al.*, 1984). The secretion has been shown to be inhibited by M_3 antagonists and partially inhibited by M_1 antagonists (Mullol *et al.*, 1992a). Methacholine is often used for nasal challenge as an indicator of secretory response (Mygind & Dahl, 1996). Muscarinic receptors may play an important role in the increased glandular responsiveness in rhinitis, since subjects with rhinitis demonstrated hyperresponsiveness to methacholine (Druce *et al.*, 1985; Devillier *et al.*, 1988; Stjärne *et al.*, 1989). An increase in the number of muscarinic receptors may be responsible for an increase in responsiveness to methacholine (Ando *et al.*, 1989; Ishibe *et al.*, 1983).

VIP is co-localized with Ach in parasympathetic nerves in the nose (Uddman & Sundler, 1986; Klassen *et al.*, 1988; Baraniuk *et al.*, 1990b). When released from parasympathetic nerves, it acts upon binding sites that are present on the epithelium, glands and vessels in human nasal mucosa (Baraniuk *et al.*, 1990b). Nerve fibres containing VIP are richly distributed in the nasal mucosa. The fibres are particularly numerous around small blood vessels. They can also be seen around submucosal glands and beneath the surface epithelium. The distribution of its binding sites and

nerve fibres indicates a role for VIP as a vasodilator and secretagogue. VIP is a more potent vasodilator than Ach, it dilates both resistance and capacitance vessels in nasal mucosa (Malm *et al.*, 1980). *In vitro*, VIP has been shown to stimulate glandular secretion from human nasal mucosal explants (Mullol *et al.*, 1992c). *In vivo*, VIP may be more active as a vasodilator than as a secretagogue (Malm *et al.*, 1980; Lundberg *et al.*, 1981; Stjärne *et al.*, 1991). Alteration of VIP innervation may occur in rhinitis since an increase in VIP immunoreactive material and an increase in VIP tissue concentration have been reported (Fang & Shen, 1998a; Fang *et al.*, 1998b).

1.6.3 Sensory nerves

In addition to the sympathetic and parasympathetic systems, the response of nasal structures to external stimuli may be mediated through activation of sensory nerve fibres. The nasal sensory nerves originate in the trigeminal ganglion and innervate the nose via the ethmoidal and posterior nasal nerves. Sensory nerves detect the conditions of inspired air and respond to mucosal injury, noxious mechanothermal and chemical stimuli by conveying messages of injury to the central nervous system, and by initiating local vascular and glandular responses. The sensory fibres densely innervate arterial and venous vessels. They are present in submucosal gland acini and in interstitium and extend up to the epithelium (Uddman & Sundler, 1986; Baraniuk & Kaliner, 1990b; Baraniuk *et al.*, 1991a; Baraniuk & Kaliner, 1991b).

Sensory neurones contain multiple neuropeptides such as the tachykinins substance P, and neurokinin A (NKA) and calcitonin gene-related peptide (CGRP), which are co-localised and appear to be co-released upon depolarization of the nerve ending. In addition to external stimuli, many inflammatory mediators can stimulate sensory nerves to cause depolarization and the release of neuropeptides. The local release of neuropeptides constitutes the axon reflex. The axon reflex represents a very rapidly deployable defence mechanism and has been clearly demonstrated in rodent nasal mucosa (Lundblad *et al.*, 1984). Human nasal studies to date have not clearly demonstrated the influence of axon responses. The neuropeptides released from the

sensory nerves have their own unique actions, which are determined by the distribution of cells bearing their specific receptors. Autoradiography of CGRP, substance P and NKA binding sites has revealed that each peptide has a unique distribution of binding sites in human nasal mucosa (Uddman & Sundler, 1986; Baraniuk & Kaliner, 1990b; Baraniuk *et al.*, 1991a; Baraniuk & Kaliner, 1991b).

1.6.3.a Tachykinins: substance P and neurokinin A (NKA)

Tachykinins are a family of peptides which share the C-terminal sequence Phe-X-Gly-Leu-amidated Met. They are also called neurokinins. Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-amidated Met) and NKA (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-amidated Met) are present in sensory nerves, and are coded by the same preprotachykinin A gene. Three tachykinin receptors, NK₁, NK₂ and NK₃ have been cloned. Substance P is the preferred ligand for NK₁ receptor while NKA is the preferred ligand for NK₂ receptors. NKB (produced from preprotachykinin B gene), is the preferred ligand for NK₃ receptors (Helke *et al.*, 1990). Recently an abundant source of peripheral NKB has been found in the human and rat placenta (Page *et al.*, 2000 and 2001).

In human nasal mucosa, NKA and substance P immunoreactive nerve fibres are found in the walls of arterioles, venules and sinusoids, and are also found in the submucosal glands, basement membrane and epithelium (Baraniuk *et al.*, 1991a). However, the binding sites for radiolabelled substance P are found on arterioles, venules and glands whereas those for NKA are only on arterioles (Baraniuk *et al.*, 1991a). Substance P and NKA induced vasodilatation and vascular permeability in rhinitic subjects (Braunstein *et al.*, 1991). Substance P is likely to play a role in the nasal inflammatory process since its binding sites are widely distributed and its concentration is significantly increased immediately after nasal allergen challenge (Mosimann *et al.*, 1993). Allergic rhinitics have significantly higher tissue concentrations of substance P than normal subjects (Fang *et al.*, 1998a). Nasal provocation with substance P induced a dose-dependent increase in nasal airway resistance in both normal (Devillier *et al.*, 1988; Chatelain *et al.*, 1995) and allergic rhinitic subjects (Devillier *et al.*, 1988; Fajac

et al., 1995; Konno *et al.*, 1996). Substance P induces serous and mucous glandular secretion from human nasal mucosal explants *in vitro* (Baraniuk *et al.*, 1991c; Mullol *et al.*, 1992c). Substance P can stimulate upregulation of endothelial cell adhesion markers which may facilitate leukocyte traffic into the nasal mucosa and the development of the late phase response.

1.6.3 b Calcitonin gene-related peptide (CGRP)

CGRP is 37 amino acid residues long and is produced in two forms: CGRP- α and CGRP- β , which differ by 3 amino acids. Both forms are vasodilators. In human nasal mucosa, CGRP-containing nerve fibres densely innervate arterial vessels, but also innervate venous vessels and some gland acini (Uddman & Sundler, 1986; Baraniuk & Kaliner 1990b). CGRP binding sites are concentrated on arterioles (Baraniuk & Kaliner 1990b). No binding sites have been found on glandular cells. CGRP may be an important long-acting vasodilator and contribute to filling of venous sinusoids, and thus may induce an increase in nasal airway resistance. CGRP induces nasal obstruction and an increase in superficial blood flow in normal subjects (Chatelain *et al.*, 1995; Rangi *et al.*, 1990). *In vitro* and *in vivo*, CGRP has no effect on glandular secretion (Baraniuk *et al.*, 1990c; Guarnaccia *et al.*, 1994). These data are consistent with the CGRP binding sites on arterial vessels. CGRP therefore contributes primarily to the neural control of nasal vascular blood flow by inducing arterial vasodilatation.

1.6.4 Sensory-parasympathetic reflexes

A major result of sensory nerve stimulation in the nose is the recruitment of parasympathetic and other central reflexes, such as sneeze and sensations of itch and burning (Raphael *et al.*, 1989b and 1991b). Some trigeminal sensory nerve fibres are present in the sphenopalatine ganglion, which belongs to the parasympathetic system. This connection may produce a direct reflex between afferent nociceptive fibres and parasympathetic neurones, figure 1.9. Sensory-parasympathetic reflexes can be induced by nasal provocation with histamine (Raphael *et al.*, 1989b), capsaicin,

nicotine (Stjarne *et al.*, 1989) and oral provocation with chilli peppers (Raphael *et al.*, 1989c); they lead to coordinated vascular congestion with mucosal thickening, obstruction to airflow, and glandular secretion. The cholinergic component appears to be predominant, since atropine blocks this reflex-induced glandular secretion (Raphael *et al.*, 1989a and 1989b). The sensory-parasympathetic reflex arc is of importance in the normal nose, since it may serve to protect the nasal mucosa by inducing glandular secretion. This reflex arc may also contribute to the pathology of allergic rhinitis, vasomotor rhinitis and infectious rhinitis.

1.6.5 Neutral Endopeptidase (NEP)

Neuropeptide actions are limited by the enzyme neutral endopeptidase, NEP (Nadel, 1990; Borson *et al.*, 1991). NEP is active upon SP, NKA, CGRP, endothelin, BK and many other peptides. It is present in all cells which possess peptide receptors (Borson, 1991). In situ hybridization of NEP mRNA, and immunohistochemistry of NEP immunoreactive material have revealed NEP in epithelium, glands and vessels (Baraniuk *et al.*, 1993) which confirms the above comment. NEP plays a role in regulating neuropeptide responses. Inhibition of NEP activity by phosphoramidon potentiates the effects of SP, NKA, BK and other peptides (Borson *et al.*, 1991; Umeno *et al.*, 1989; Lurie *et al.*, 1994). Destruction of NEP or NEP-containing epithelial cells, or decreases in NEP production, may occur during pathological states. NEP activity is reduced during viral infection (McDonald *et al.*, 1988) and is destroyed by cigarette smoke (Dusser *et al.*, 1989). The release of peptides into areas with reduced NEP activity may lead to prolonged, unopposed inflammatory effects, enhanced glandular secretion and vascular permeability, and may contribute to respiratory hyperresponsiveness.

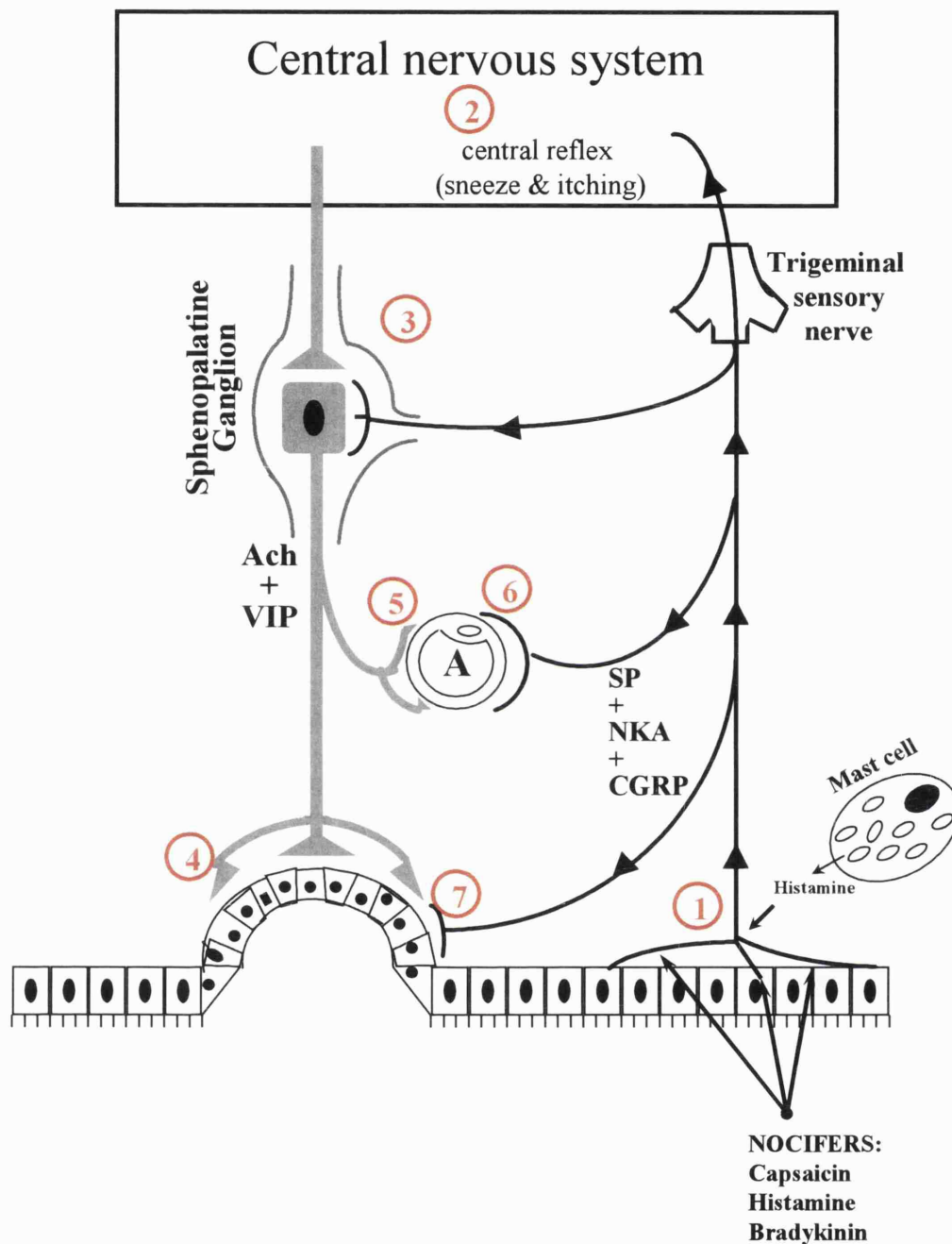


Figure 1.9: Schematic representation of sensory-parasympathetic reflexes. Stimulation of sensory nerve endings by nocifens such as capsaicin, histamine and bradykinin (1) induces a spread of action potentials in the nerve via axon reflexes. Sensory nerve stimulation leads to induction of central reflexes (2) such as sneeze, and parasympathetic reflexes (3) which lead to glandular secretion (4) and vasodilatation (5). Neuropeptides released by axon-reflex can act upon vessels to induce vasodilatation (6) and possibly glandular secretion (7), (Uddman *et al.*, 1986; Baraniuk *et al.*, 1995; Lacroix *et al.*, 1995).

1.6.6 Nerves and hyperresponsiveness in rhinitis

Neural mechanisms contribute to many nasal symptoms. Parasympathetic and sensory responses combine to increase nasal blood flow, thickening of the mucosa, induce plasma extravasation, and glandular secretion. In allergic rhinitis, one of the major pathological changes that occurs is hyperresponsiveness to daily life stimuli such as, dust, pollen and fumes. Increased responsiveness of sensory nerves (sneeze) and nasal glands (hypersecretion) is a characteristic clinical feature of rhinitis, which is responsible for the symptomatology (Andersson *et al.*, 1987; Birchall *et al.*, 1993). The symptoms and nasal sensitivity to stimuli are exaggerated during pathological states. Inflamed nasal passages demonstrate increased blockage and secretion after stimulation with BK (Baraniuk *et al.*, 1994b; Riccio & Proud, 1996), endothelin (Riccio *et al.*, 1995) and methacholine (Riccio & Proud, 1996).

Nerves and neuropeptides may have a role in the development of nasal airway hyperresponsiveness. Methacholine induces glandular secretion in both normal and atopic rhinitic subjects, however, there is twice as much mucus secreted in allergic rhinitic than normal subjects (Druce *et al.*, 1985). An increase in the number of muscarinic receptors may be responsible for a hypersecretion to methacholine (Ando *et al.*, 1989; Ishibe *et al.*, 1983). Substance P and methacholine cause a greater rise in nasal airway resistance in allergic rhinitics than in normal subjects (Devillier *et al.*, 1988). The nasal vasculature of rhinitics displays a richer innervation of VIP, CGRP and substance P than that of non-rhinitic blood vessels (Fang & Shen 1998a). The difference in nasal response to methacholine and substance P, and an increase in sensory nerve innervation in rhinitic subjects suggests a role for sensory and parasympathetic responses in pathological states. There is an increase in sensory-parasympathetic reflexes-induced glandular response to bradykinin provocation in severe perennial allergic rhinitics than in normal subjects (Baranuik *et al.*, 1994b; Riccio *et al.*, 1996).

Immunocytochemistry showed that in the human nasal mucosa of chronic rhinitics, substance P terminals were adjacent to, or have direct contact with, mast cells (Zhao *et al.*, 1995). Substance P induces histamine release from human nasal mast cells *in vitro* (Schierhorn *et al.*, 1995). However, a number of *in vivo* studies were unable to demonstrate histamine release from human nasal mucosal mast cells (Braunstein *et al.*, 1991 and 1994). It is possible that the absence of histamine following exogenous substance P may be due to the low concentrations used or that substance P may be readily degraded in nasal mucosa.

1.7 METHODS FOR STUDYING THE ROLE OF INFLAMMATORY MEDIATORS IN HUMAN AIRWAY

For the study of the human nasal airway a nasal challenge or nasal provocation test involving the administration of allergens or a test substance is employed in order to determine the nasal response. The nose has a limited range of responses to nasal challenge, which are, change in the nasal patency, induction of secretion, itching and sneezing. The severity of the response to nasal challenge may be measured by several techniques which enable us to monitor each component of the nasal response. Such techniques include:

- assessment of subjective appreciation of nasal symptoms
- assessment of nasal resistance to airflow
- collection of nasal secretion
- lavage of nasal airway

Assessment of subjective appreciation of nasal symptoms can be done by symptom scores. In symptom scores, the sensation of the change in nasal response, such as nasal obstruction, itching and sneezing are recorded using visual analogue scales (Aitken *et al.*, 1969) or simple numbers. Symptom scores have been extensively applied in clinical studies of rhinitis therapy. It is a reasonable technique to assess

nasal response, however, this technique should be complemented by at least one other because of its inherent variability.

The assessment of nasal resistance to airflow provides a more accurate measurement of the degree of nasal obstruction. Nasal resistance to airflow is primarily governed by the state of congestion of blood vessels in the nasal passages, which are situated at the anterior end of the inferior nasal turbinate. There are several methods for the assessment of nasal airway resistance, these are rhinomanometry (Kern *et al.*, 1973), acoustic rhinometry (Jackson *et al.*, 1997) and nasal peak flow (Taylor *et al.*, 1973). In the rhinomanometry technique, the airflow through the nasal cavity and the pressure difference across the nasal airway are measured. By applying Ohm's Law, the nasal resistance to airflow can be calculated. Acoustic rhinometry involves the measurement of a sound wave reflected back from the nasal passage. The sound wave is usually generated by a spark generator, and the wave is directed towards the nose through a connecting tube. A microphone on the tube detects the wave reflected back from the nasal passage, and sends this signal for analysis of data. The data are displayed on the computer screen as a graph of nasal cross-sectional area against distance into the nasal cavity.

Induction of secretion is one of the main responses to different challenges. The change in the amount of secretion reflects secretory activity of the nasal mucosa. Collection of secretions after challenge is not usually a problem. The problem arises when some sort of baseline, pre-challenge secretions are required. Several methods have been suggested for obtaining amounts of secretion, these will be discussed in chapter 2, section 2.2.4.

Nasal lavage is one of the most useful procedures, since it enables the substances released into the nasal cavity to be quantified. Such substances include mediators and proteins, released following nasal challenge. By identifying mediators in the nasal lavage, one could identify which mediators may play a part in the pathogenesis of rhinitis. Identification of proteins in nasal lavage can give information on the mechanisms involved in the nasal responses; congestion and rhinorrhea. For example,

an increase in albumin concentration in nasal lavage indicates increased vascular permeability. Lavage techniques have been performed in a variety of ways, but the basis of the techniques involves the instillation of saline into the nasal cavity and recovering the return lavage.

Each technique therefore provides a study of different components of the nasal response. In this study, the secretory activity and the source of protein in nasal secretion are investigated.

1.8 AIMS OF THE WORK

Many mediators and neurotransmitters have a role in the development of nasal airway disease. The processes of nasal disease are complex, however, many different mediators and neurotransmitters, at the end, produce a similar end response such as nasal blockage, rhinorrhea and nasal irritation. The aim of this project was to analyse quantitatively the secretory responses of the human nasal mucosa. The subsequent chapters will present data from investigations into the following:

- Using normal human subjects, the effect of difference dose of methacholine, histamine and bradykinin on the secretory response of the nasal mucosa was investigated. By examining the amount of secretion and the concentrations of lactoferrin, lysozyme, albumin and fucose, in nasal lavage following nasal provocation with each mediator, the mechanism of action that gives rise to secretion will be described.
- The role of histamine H₁ and bradykinin B₂ receptors in the secretory response will be investigated by studying the effect of the histamine H₁ receptor antagonist, cetirizine, and the bradykinin B₂ receptor antagonist, Hoe 140, on the histamine- and bradykinin-induced secretory responses in normal human subjects.

- Histamine has been proposed to induce a neuronal reflex in the human nasal mucosa. The effect of unilateral challenge with 100, 300 and 1000 μg histamine on the amount of secretion and the protein content was investigated, in order to examine the extent of histamine-induced neuronal reflex in the human nasal mucosa.
- There is growing evidence that substance P is one of the main neuropeptides involved in allergic rhinitis. Using normal and atopic subjects the effect of substance P on the secretory response and the protein content in nasal lavage was investigated.
- These approaches aim to provide an insight into the type of nasal secretion (e.g. mucous or serum) that result from the action of a variety of established inflammatory mediators. The patterns of secretion resulting from the effects of different mediators will also be compared.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 SOURCES

Table 2.1: The materials used in this study and their sources are listed below.

Material	Source
Acetyl- β -Methacholine chloride	Sigma Chemical Co., UK
Albumin standard	Sigma Chemical Co., UK
Bradykinin	Peninsula, UK
Carbonate buffer	Sigma Chemical Co., UK
Cetirizine	UCB Pharmaceuticals Ltd., Belgium
Cotton buds	Boots company PLC, UK
Cotton pads	Boots company PLC, UK
Cysteine reagents	Sigma Chemical Co., UK
Ethanol	BDH Laboratory Supplies, UK
Goat antihuman serum albumin conjugated to horseradish peroxidase	Bethyl Laboratories Inc, USA
Goat serum	ICN Biomedicals Inc, USA
H ₂ SO ₄	Merck, UK
Histamine	Sigma Chemical Co., UK
Hoe 140 (Icatibant)	Hoechst AG, Germany
Human serum albumin	Sigma Chemical Co., UK
Lactoferrin standard	Sigma Chemical Co., UK
Lysozyme standard	Sigma Chemical Co., UK
Methylpentose	Sigma Chemical Co., UK
Micrococcus lysodeikticus	Sigma Chemical Co., UK

NaOH	Sigma Chemical Co., UK
<i>o</i> -phenylenediamine dihydrochloride substrate	Sigma Chemical Co., UK
Phosphate buffer saline system kit	Sigma Chemical Co., UK
Placebo	UCB Pharmaceuticals Ltd., Belgium
Polypropylene microtiter plates	Becton Dickinson Labware, UK
Rabbit anti-human lactoferrin	Dako Ltd., UK
Rabbit anti-human lactoferrin-horseradish peroxidase conjugate	Dako Ltd., UK
Substance P	NOVA Biochem Co., UK
Tween 80	Sigma Chemical Co., UK
Whatman filter paper	Whatman, UK

2.1.2 DILUTION OF NASAL CHALLENGE AGENTS

Sterile saline (NaCl, 154 mM) was used for making up both stock solutions and their subsequent dilutions. All solutions were made up under sterile conditions in a class 2 microbiological safety cabinet. Histamine, as the diphosphate salt, was dissolved to make a 10 mg/ml stock solution. This stock solution of histamine diphosphate was diluted with sterile saline to obtain 3 mg/ml and 1 mg/ml solutions for experimental use. Acetyl- β -methacholine chloride (methacholine) was dissolved in sterile saline to make a stock concentration of 100 mg/ml, which was reserved for experimental use and for subsequent dilution to make 30 mg/ml and 10 mg/ml solutions. Histamine and methacholine solutions were stored at 4°C, and were allowed to achieve room temperature before application.

Bradykinin and substance P were dissolved to make stock solutions of 10 mg/ml and 1 mg/ml respectively, which were aliquoted in an appropriate amount and stored at -20°C. When used, bradykinin and substance P stock solutions were diluted in sterile saline to achieve concentrations from 3 mg/ml to 0.1 mg/ml and 0.5 mg/ml to 0.1

mg/ml respectively. Hoe 140 was dissolved to make a stock solution of 10 mg/ml which was also divided into aliquots and stored at -20°C. An aliquot of Hoe 140 was diluted to 2 mg/ml for experimental use. Bradykinin, substance P and Hoe 140 aliquots were diluted to their required concentrations immediately before use.

The challenge solutions were delivered using a normal pump spray which delivers 100 µl per activation.

2.1.3 SUBJECTS

For all studies, except in chapter 6, normal, healthy volunteers aged 20 to 60 years were used. No subject was taking any medication at the time of the experiments or within the week prior to experiments. For the study in chapter 6 involving atopic subjects, selection was made on the basis of a clinical history of either seasonal (4 subjects) or perennial (3 subjects) allergic rhinitis; the subjects taking part were within the age range, 20 to 30 years. None of the subjects had asthma. All subjects were off all oral and intranasal therapy for two weeks prior to the study. All subjects gave informed consent and the studies were approved by the local Ethics committee at University College London. Experiments were performed in a laboratory with a controlled temperature of 20°C.

2.2 METHODS

2.2.1 NASAL CHALLENGE

Methods described for introducing challenge agents into the nasal cavity have included aerosol administration and application of a filter paper disc saturated with challenge solution to the inferior turbinate. The aerosol administration, using a nasal pump spray (Perfect-Valois, UK Ltd.) was chosen for nasal challenge as this method can deliver the challenge solution throughout the nasal cavity. The spray delivers 100

μl per activation with 98% accuracy. The device was placed in one nostril and activated once, then repeated for the opposite nostril.

2.2.2 RECORD OF SYMPTOMS

The symptom scores method, where subjects were asked to score values of severity of symptoms, was not applied in this study, as it is a subjective measurement. However the number of sneezes was counted and recorded after every challenge. Any other symptoms, such as itching, pain and sore throat, experienced were given voluntarily by the subjects and noted.

2.2.3 NASAL LAVAGE

Nasal lavage enables the collection of a small amount of secretion with minor perturbation of the nasal mucosa. It is a relatively non-invasive technique, which is well tolerated by volunteers, and has many additional advantages including ease of performance, reproducibility and sampling of the mucosa at, or close to, the site of inflammation. The technique involves the instillation of lavage fluid into the nasal cavity and allows for the collection of fluid, which can be analysed for either mediators of inflammation or the presence of inflammatory cells and proteins.

The method used in this study was described by Wihl *et al.* (1995). The technique is easy to perform, gives a recovery of approximately 70-90% of fluid and is well tolerated by subjects. Each nasal cavity was lavaged separately with 5 ml sterile saline at 37°C. The test subject, in a sitting or standing position, leaned forward at an angle of about 45°-60°. This position prevented fluid from reaching the throat. The 5 ml of fluid was delivered into the nasal cavity through the use of a 10 ml syringe to which nasal olives were attached to cover the apex of the nasal cavity and thus prevent any leakage of fluid. The lavage fluid was passed slowly into the nasal cavity and back into the syringe three to four times before collection of the lavage fluid. Figure 2.1

illustrates a subject undergoing the lavage technique. In each experiment, three initial washes were made in order to remove any pre-existing substances, and the third lavage was reserved and used as the baseline. The lavage fluid was then centrifuged at 4°C for 10 minutes at 1000g. The supernatant was divided into appropriate amounts in microcentrifuge tubes, ready for biochemical analysis for lactoferrin, lysozyme and albumin. Two ml of the pellet was pipetted into a test tube and reserved for mucous glycoprotein assay. All the reserved lavages were stored at -70°C until assay.

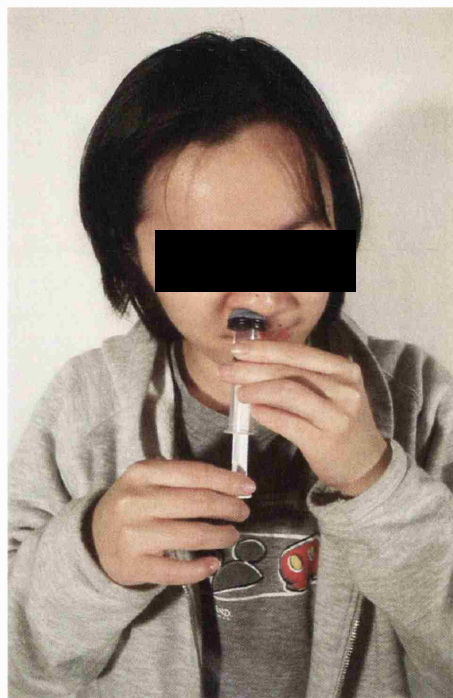


Figure 2.1: Test subject in nasal lavage technique.

2.2.4 QUANTIFICATION OF NASAL SECRETION

The production of nasal secretion is influenced by many factors including ambient temperature and humidity, airborne irritants, and allergic nasal disorders. Many studies have been carried out to analyse the content of nasal secretions by means of

nasal lavage. However, the lavage technique only provides information about the composition of the nasal secretion and not the nasal secretory activity. The amount of nasal secretion reflects the secretory activity of the nasal mucosa. A variety of different techniques have been performed in order to assess the effect of the challenge solution in inducing nasal secretion. The techniques include:

1. blowing the nose, before and after nasal challenge, on preweighed tissue (Holmberg *et al.*, 1990)
2. collecting drips of nasal secretion from the apex of the nose (Borum *et al.*, 1979)
3. insertion of preweighed filter paper disks (Naclerio *et al.*, 1992b)
4. insertion of preweighed filter paper strips (Lorin *et al.*, 1972; Knowles *et al.*, 1981)

The technique of blowing the nose and the collection of drips eliminates irritation and sneeze triggered by the insertion of any matter into the nasal cavity. However, these methods cannot detect a small amount of nasal secretion such as baseline secretion. In addition, the concentration of challenge solution used in both techniques must be higher than other techniques in order to produce enough secretion to blow out of the nose or to drip from the apex of the nose. High doses of challenge solution may cause unwanted systemic side effects in subjects. Furthermore, these techniques cannot be used to study the effect of unilateral nasal challenge since the secretion cannot be collected separately.

Although the insertion of filter paper disks technique reduces the chance of irritation and sneezing, and a low concentration of challenge solution is enough to induce detectable secretion, a disk has a limited capacity, and the technique requires a lot of expertise in order to place, or remove the disk, on, or from, the required site. Moreover the secretion collected comes from only one particular site in the nose.

The filter paper strips technique was considered, since small amounts of secretion can easily be assessed. Also, a lower concentration of challenge solution is enough to induce detectable secretion. The filter paper strips technique is simpler than the disk

technique since the strip can rapidly and easily be inserted and removed. Cotton buds were another material considered in these studies since they have the same advantages as the filter paper strips. Preliminary experiments were carried out in order to determine which materials, filter paper or cotton buds would be used, and the protocol to be utilised in the studies.

2.2.4.1 Justification for the technique to measure nasal secretion quantitatively.

The study in this section was designed to evaluate a simple technique for assessing nasal secretory activity (amount of nasal secretion). Two separate experiments were carried out to evaluate the use of both filter paper and cotton bud on baseline secretion, and the second experiment was to compare the rate of absorption of both materials.

2.2.4.2 Materials

Subjects

Six normal healthy adult subjects aged between 20 to 60 years of age were studied after informed consent was obtained. Subjects were not studied within 3 weeks after recovery from upper respiratory tract infections, and no subjects complained of nasal symptoms at the time of study. No medication was taken within a week prior to the experiment.

Materials

100% pure cotton buds were obtained from Boots Company PLC, Nottingham, England. Whatman No. 1 filter paper 5.5 cm in diameter was cut in half. Each half was folded into a flat triangle. The triangle was folded in half forming a V shape when looking at the base, figure 2.2. For each insertion the tip of the triangle was in the nostril while the V base protruded from the nostril.

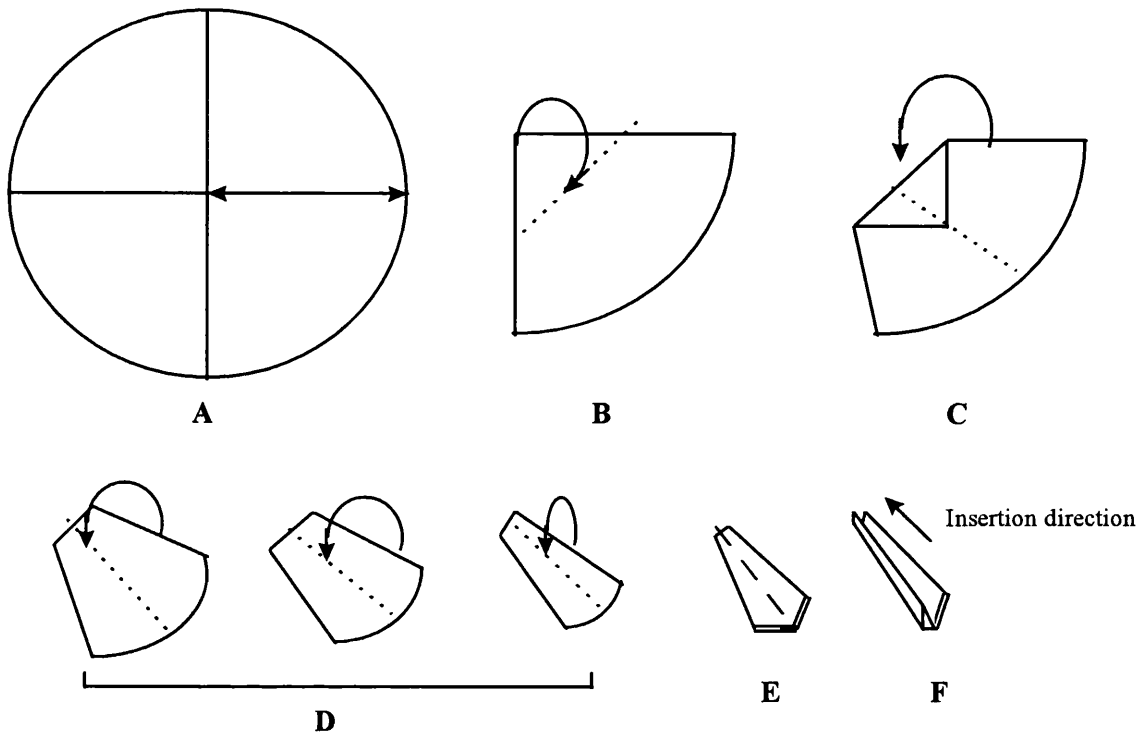


Figure 2.2: Folding of filter paper.

2.2.4.3 Methods

Base line secretion measurement protocols

Two protocols were employed in the baseline secretion study. The first protocol involved the repetitive insertion of filter papers and cotton buds. Prewighed filter paper strips or a cotton bud was inserted into the nose, one in each nostril, and left in place for 2 minutes before removal. Immediately following removal, the 2nd pair of preweighed, filter paper strips or cotton buds were inserted. The same routine was carried out for 20 minutes with a total of 10 insertions for each nostril. After 2 minutes absorption the filter papers or cotton buds were returned to their preweighed

test tubes and reweighed. Four subjects underwent both experiments and there was at least a 24 hour gap between each experiment.

In the 2nd protocol the same procedure was performed on 6 subjects but between each measurement an 8-minute gap was allowed before the next insertion of filter papers or cotton buds. Each experiment took 1 hour with a total of 7 insertions in each nostril.

Comparison of absorption rate of the two materials.

The study was carried out in order to compare rate of absorption by filter paper strips and cotton buds. Two sets of experiments were conducted, one with 2 minutes absorption and the other with 5 minutes absorption. At least 2 ml of nasal secretion was obtained from an atopic volunteer with a history of hypersecretion, by inducing irritation of the nasal mucosa. This was carried out by insertion of a large rectangular strip of filter paper into each nostril, which was left in place until secretions started to drip and the required amount of secretion (2 ml) was collected. The secretion was divided into 0.2 ml aliquots and added to a Bijou tube. Five filter papers and cotton buds were preweighed in test tubes. Each filter paper and cotton bud was placed in a Bijou tube containing 0.2 ml secretion. In the 1st set, cotton buds and filter papers were allowed to absorb the secretion for 2 min and were then removed and reweighed. Five minutes absorption was allowed in the 2nd set. The mean rate of absorption was calculated by taking the mean of 5 samples.

Statistics

The data were analysed by non-parametric statistical tests using SigmaStat Software version 2.0. The Wilcoxon signed rank test was used to assess differences between the absorbed secretion of the two materials, by comparing the values of area under the curve of secretion absorbed (AUC), for each 20 minutes, and 1 hour period of observation. In the comparison of absorption rate study, the Wilcoxon signed rank test

was used to determine the significance of the difference between the filter paper strips and cotton bud absorption rate. A p-value of ≤ 0.05 was considered to be significant.

2.2.4.4 Results

All subjects had difficulty inserting a filter paper strip and all of them found it painful compared to a cotton bud. Lacrimation was observed more frequently in filter paper-collections (all subjects) than cotton bud-collections (2 out of 6 subjects). Five out of six subjects sneezed after insertion of filter paper. Application of both materials produced a soreness of the nasal mucosa after the 3rd or 4th insertion. Furthermore, the nasal secretion collected by filter papers, over a 20-minute period, was higher than that of cotton buds (Wilcoxon test, p-value < 0.05). The baseline obtained from the use of filter papers was not as steady as that of cotton buds, figure 2.3 (A). The same difference between the two materials was also observed in the 2nd protocol, where a resting period of 8 minutes was allowed, (Wilcoxon test, p-value ≤ 0.05), figure 2.3 (B). However, the secretion curve for the filter paper strip in B was steadier and the amount of secretion obtained was lower in B than in A. No complaint of soreness of the nasal mucosa was expressed with subsequent insertions in the 2nd procedure.

In order to assess whether the high amount of the secretion collected by filter paper was due to a higher absorption rate or extra secretion, another study was conducted to compare the rate of absorption. It was found that cotton buds have a higher absorption rate than filter papers, Wilcoxon test, p-value < 0.05 (Figure 2.4).

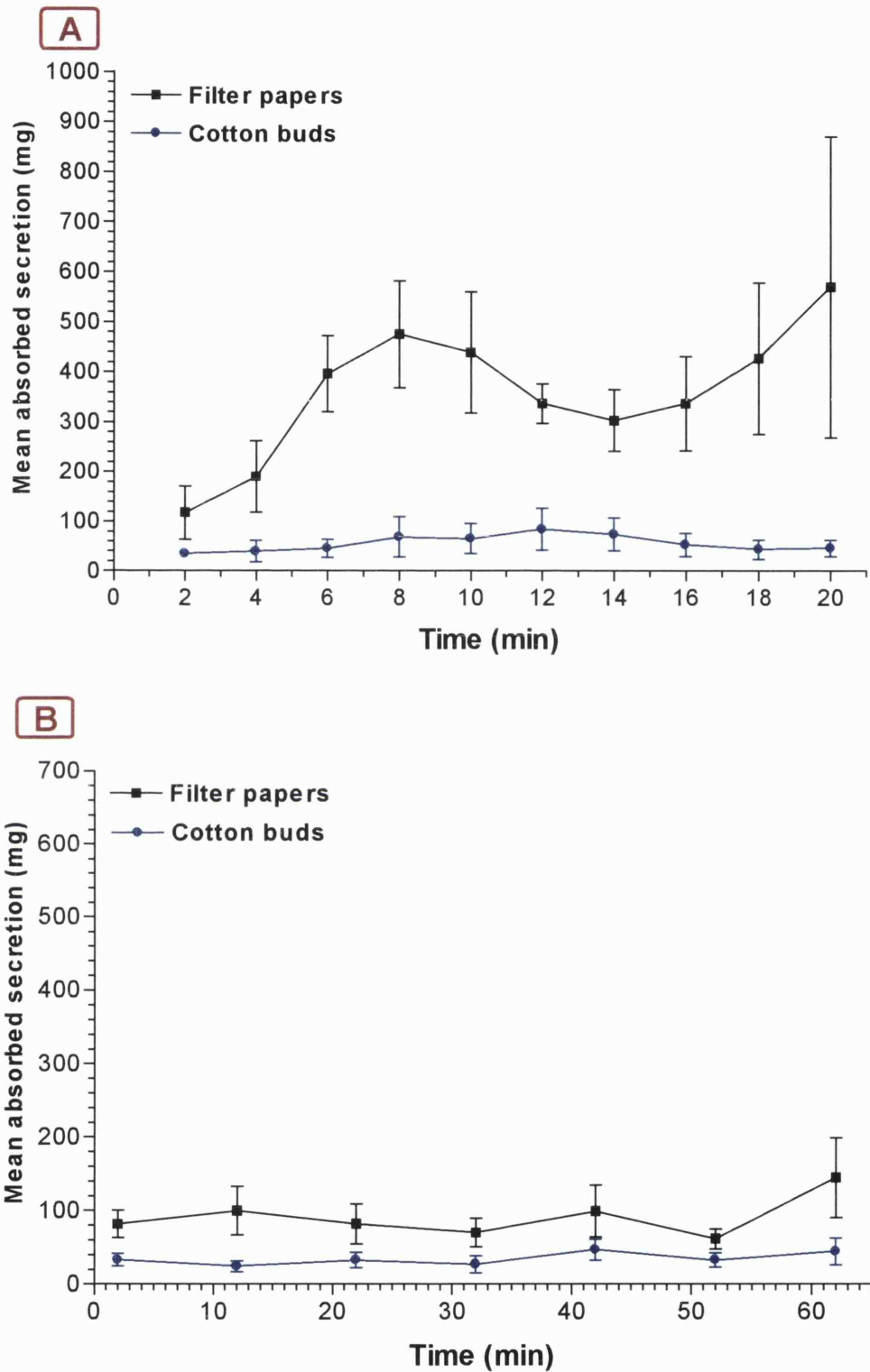


Figure 2.3: The graphs representing the nasal secretion absorbed using filter paper strips (■) and cotton buds (●). The data are means \pm S.E.M. In (A) filter paper strips and cotton buds were inserted repetitively every 2 minutes. In (B) eight minute gaps were allowed between each insertion of filter paper strips and cotton buds. In both protocols, filter paper and cotton buds were left in the nostril for 2 minutes.

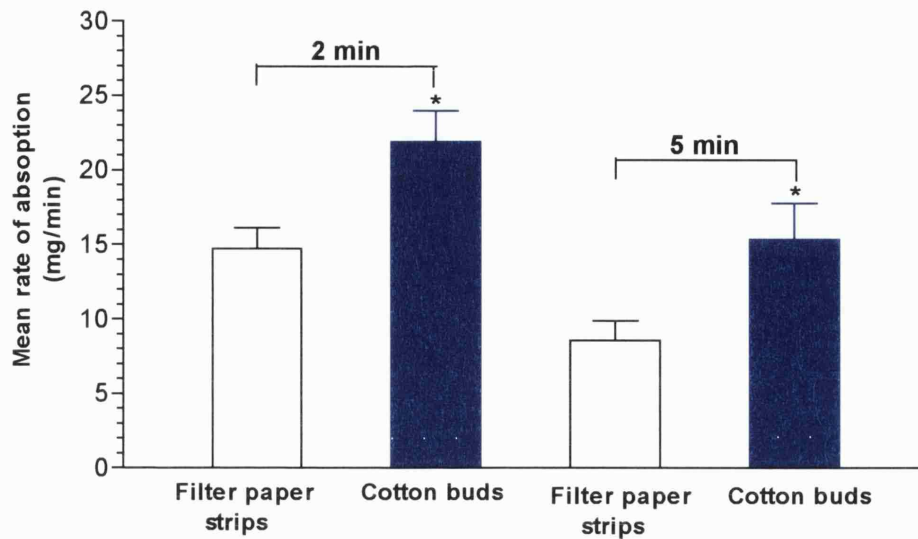


Figure 2.4: Mean rate of absorption (mg/min) \pm S.E.M. of filter paper strips and cotton buds with 2 minutes and 5 minutes absorption time. Cotton buds have a higher absorption rate than filter paper strips; * p-value < 0.05.

2.2.4.5 Discussion

Looking at figure 2.3 (A) and (B), they indicate that the high secretion obtained from filter paper strips in figure 2.3 (A) was due to irritation or soreness of the nasal mucosa, induced by repetitive insertion, since a smaller and steadier secretion was obtained when a resting period was allowed between each insertion (figure 2.3 (B)). When comparing the use of the two materials, the higher amount of secretion obtained with filter paper may be due to greater irritation, induced by insertion of the filter paper strip, since it was demonstrated that the cotton bud has a higher absorption rate than filter paper (figure 2.4). The reduced rate of absorption at 5 minutes is likely to be due to saturation. Cotton buds produced a steady base line, and the incidence of lacrimation and sneeze was less. Cotton buds contain pure cotton, while the filter paper may also contain other material, which could explain why filter paper causes more irritation than cotton buds. In addition, cotton buds are softer than filter paper and so are easier to insert without causing pain and irritation. By taking this into account, the use of cotton buds has several advantages over that of filter paper,

therefore they were used as a material for the study of nasal secretory response to nasal challenge.

This study has shown that cotton buds can be used in the assessment of secretory activity of the nasal mucosa. Furthermore, the experiment showed that insertion of filter papers and cotton buds, repetitively, induced more secretion and caused soreness. Therefore, in future experiments there should be a resting period between each insertion. The capacity of cotton buds can be increased by wrapping a thin cotton pad around the middle stem so that a higher amount of secretion, induced by the challenge solution can be collected.

2.2.5 LASER DOPPLER FLOWMETER.

Cutaneous blood flow of the facial skin was recorded, in the study with substance P, chapter 6, by the use of a laser Doppler flowmeter (model MBF2, Moor Instruments, England). The laser Doppler flowmeter provided a power output at the probe of about 1nW and used an infra-red laser of wavelength 780 nm. The laser Doppler flow probe measures red cell flux at a specific site at a depth of approximately 1 mm. The measurement of the red cell flux at such a site is represented as the height (cm) of the peak on a chart recorder. The changes in red cell flux, corresponding to the change in blood flow, can then be obtained by finding the difference in the height of the peaks (Izumi *et al.*, 1991). The probe was placed on the cheek and was held 1 inch from the base of the nose. Recordings were made at the same site prior to nasal challenge and after the challenge.

2.2.6 BASIC PROTOCOL

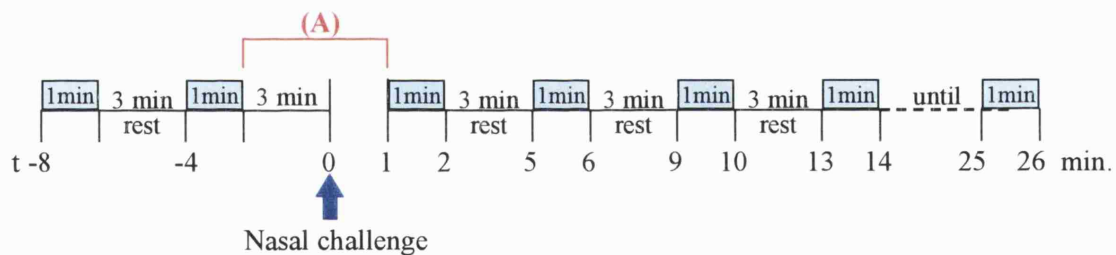
Details of the protocol for each experiment are described in each chapter. The following protocols are common to all studies.

2.2.6.1 Basic procedure for measuring nasal secretory response.

Following the preliminary experiments, cotton buds were selected as the material to be used, and a resting period between each insertion was allowed. An observation period of 26 minutes was chosen based on preliminary experiments. However, an 8-minute resting period is too long when one wants to study the time course of a challenge solution within 26 minutes. From preliminary observations, a resting time between each insertion of 3 minutes was sufficient to avoid irritation, and it did not affect the baseline measurement of nasal secretion. Therefore, the resting period between each insertion was reduced to 3 minutes, so that a time course could be studied over a 26-minute period with more insertions within the study period. In order to have more insertions within the observation period, the absorption time was also changed from 2 minutes to 1 minute. The change in absorption time to 1 minute did not affect the secretion obtained since the rate of absorption into a cotton bud is still the same i.e. the absorption rates of a cotton bud over 2 minutes or 1 minute are the same. The 1 minute absorption time is still within the cotton bud's absorption capacity whereas absorption times higher than 2 minutes may alter the absorption ability due to saturation, as shown in figure 2.4. Therefore in each measurement a cotton bud was placed in the nostril for 1 minute and 3 minutes resting time was allowed between each insertion except when the challenge solution was administered.

This change in the protocol enabled the time course of the challenge solution on nasal secretory activity over 26-minute period to be followed. For all measurements of nasal secretion the common procedure was as follows. The subject was seated comfortably in an upright position. A preweighed cotton bud was inserted, prior to the nasal challenge, into each nostril, in order to absorb secretion that was already there, and any secretions that had accumulated in the nostril. A 3 minute rest was allowed

without any administration of drugs. After 3 minutes, a preweighed cotton bud was inserted for 1 minute into each nostril ($t = -4$). The amount of secretion obtained at this stage was taken as pre-challenge secretion. After a 3-minute resting period, a challenge solution was administered into each nostril ($t = 0$), 1 minute was allowed to elapse before the insertion of a preweighed cotton bud for 1 minute ($t = 1 - t = 2$), then the cotton buds were removed and reweighed. Three minutes were allowed to pass before the next insertion (figure 2.5). A total of seven insertions were performed over 26 minutes following the challenge, making nine insertions in total. After each removal, the cotton bud from each nostril was immediately reweighed: the difference in the weight reflecting the amount of secretion. The weight of the secretions from the two cotton buds was added, except for the study in chapter 5, and used for further analysis. Between each insertion, a 3-minute gap was allowed, except for the time between the pre-challenge and post-challenge measurement, where the total time is 4 minutes: (A) in figure 2.5.



1min = 1 minute absorption of secretion by means of a cotton bud

Figure 2.5: Protocol for studying the effect of challenge solution on nasal secretion.

2.2.6.2 Basic nasal lavage protocol

In the studies involving nasal lavage, the subject underwent three lavages initially followed by the nasal challenge. A single lavage was performed in each nostril, 10 minutes after the nasal challenge. The 10 minutes collection time for lavage was chosen based on a previous study (Dear *et al.*, 1995). The third lavage prior to nasal challenge and the one obtained 10 minutes after nasal challenge were reserved and

used for biochemical analysis. The lavage from each nostril was pooled prior to analysis, except for the studies in chapter 5. The lavage recovery in all studies was approximately 80-90% of the fluid.

2.2.7 DATA AND STATISTICAL ANALYSIS

Data analysis and statistical evaluation in each chapter are as described below.

2.2.7.1 Amount of nasal secretion analysis

The amount of secretion obtained from each insertion was expressed as secretion amount per minute (mg/min). This value was obtained by finding the difference in weight of a cotton bud before and after insertion, and by dividing this difference by the sum of the absorption and resting times. Once the value of the secretion per minute was obtained at each time point, the value (mg/min) was plotted against time (min). The graph achieved from such a plot shows the time course of the nasal secretory response after nasal challenge. The area under the curve (AUC) of the time course (TC) graph of each challenge solution and individual were calculated, the AUC values were used to construct a dose-response curve of a studied challenge solution.

2.2.7.2 Amount of lactoferrin, lysozyme, albumin and mucous glycoprotein.

The concentration of each protein in nasal lavage was obtained from specific assays, see section 2.3. Once the amount of the protein is obtained it is expressed as concentration in μg per 1 ml.

2.2.7.3 Data presentation

All data are presented as median values of the response with the 25th and 75th percentile values. For the secretory response at each time point over 26-minute period

of observation, the medians of the responses with 25th and 75th percentile were plotted against time (min). Biochemical analysis data and the secretory response over 26-minute periods, which expresses as the area under the curve, are presented in a dose-response plot. In chapter 6, the comparison of the responses between normal and atopic subjects are presented in a box and whiskers plot. The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers extend down to the smallest value and up to the largest.

2.2.7.4 Statistical analysis

Non-parametric statistics were employed in all studies using SigmaStat software version 2.0, since the effects were not normally distributed. A Friedman repeated measures analysis of variance on ranks was performed to see if a single group of individuals was affected by a series of different doses of challenge solution and saline. Each individual received all doses of challenge solution including saline. Dunn's test was then performed for multiple comparisons versus control (saline) treatment to determine exactly which doses of challenge solution produced significantly different effects from control (saline). A probability value (p-value) of less than or equal to 0.05 was regarded as significant.

When two paired data sets were compared, Wilcoxon signed rank test was applied. In all cases it is the difference between the same dose of challenge solution with different pretreatments. Mann-Whitney rank sum test was used when unpaired data set were employed, in this case the comparison between normal and atopic subjects. Bonferroni's correction factor was applied to adjust the p-value. Therefore, a p-value of less than or equal to 0.017 was regarded as significant.

2.3 BIOCHEMICAL ASSAYS

Nasal lavages obtained from each experiment were used to identify the presence of albumin, lysozyme, lactoferrin and mucous glycoprotein in nasal secretions. The changes in concentration of such proteins from the saline baseline indicate the source of proteins that participate in the production of secretion following nasal challenge. An increase in albumin concentration reflects increases in vascular permeability. On the other hand, measurement of the antimicrobial proteins, lysozyme and lactoferrin, both of which are secreted selectively from the serous cells in the submucosal glands, were used to indicate the serous cell activation. Goblet cells and mucous cells of the submucosal glands secrete MGP upon activation; therefore, an increase in MGP levels would indicate secretion by goblet cells and mucous cells.

2.3.1 ALBUMIN MEASUREMENT

Albumin content in the lavage samples can be measured by several techniques. However, a direct antigen competitive enzyme linked immunosorbent assay (ELISA) described by Raphael *et al.* (1988) was chosen over other techniques. This is because the assay is sensitive, reproducible, economical and can be completed in a few hours using plates which have been sensitised overnight. In direct antigen competition, two antigens are trying to bind to labelled antibody, in this case the antigens are coated albumin and soluble albumin in samples or standard albumin. The labelled antibody is antihuman serum albumin conjugated to horseradish peroxidase (HRP). An *o*-phenylenediamine dihydrochloride substrate (OPD) was used as a chromogen, and 1M sulphuric acid (H_2SO_4) was a stopping reagent. The detail of the assay is as follows (figure 2.6).

Stage (i): Human serum albumin (coated albumin), 5 $\mu\text{g}/100\ \mu\text{l}$ in 0.1 M carbonate buffer at pH 9.6, was plated overnight at 4°C in polypropylene microtiter plates.



Stage (ii): The plate was then washed 4 times with PT (0.05% Tween 80 in phosphate buffered saline (PBS), pH 7.4)

Stage (iii): The wells were blocked with 200 μ l of 1% goat serum diluted in PT, and were incubated for 30 minutes at 23°C.

Stage (iv): The plate was washed again 4 times with PT.

Stage (v): 50 μ l albumin standard or diluted samples (soluble albumin) and 50 μ l goat antihuman serum albumin-HRP, diluted 1:1000 in PT were added simultaneously into the wells. The plate was incubated for 90 minutes at 23°C.

Stage (vi): After the incubation the plate was washed 4 times with PT.

Stage (vii): The chromogenesis reaction was developed with OPD.

The chromogenesis reaction was stopped with 1M H_2SO_4 and the plate was read at 492 nm.

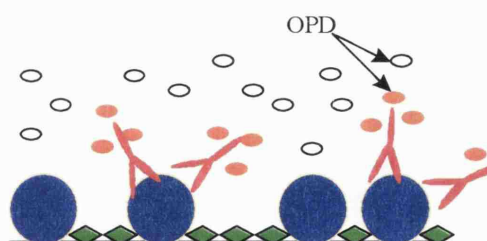
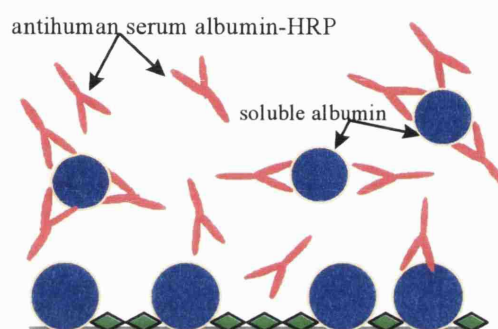
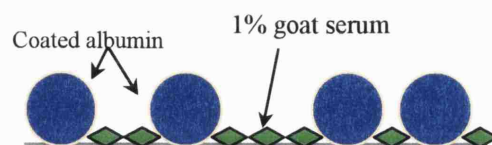


Figure 2.6: Direct antigen competitive ELISA of albumin. The reaction of standard albumin or albumin in samples (soluble albumin) with goat antihuman serum albumin-HRP (a labelled antibody) blocks the antibody from binding to the coated albumin. The more soluble albumin, the less antibody binds to the coated albumin, thus after washing the colour development will be less.

Albumin standards at different concentrations were used on every plate to construct a standard curve. The amount of albumin in the samples was generally above the detection limits and so the samples were diluted 1:2 in PT (0.05% Tween 80 in PBS,

pH 7.4) prior to an assay. Two replicates of albumin standards and each diluted sample were assayed. The intra- and inter-assay coefficients of variation were 5.8% and 34%, respectively. The values of the difference in absorbency, which are obtained from subtracting the absorbency at 492 nm for each well containing standard albumin (OD) from that of a blank well, i.e. no albumin standard or sample (OD₀), were plotted against the logarithmic value of the standard albumin concentration. The curve was fitted with a second order polynomial equation, $Y=A + B*X + C*X^2$ where A, B and C are constant values (figure 2.7).

The curve was fitted by the use of GraphPad Prism software which also gives the value of the constants A, B and C. The logarithmic value of albumin concentration in the samples could then be obtained from either direct reading from the standard curve or solving the second order polynomial equation, which is

$$X = \frac{-B + \sqrt{B^2 - 4 * C(A - Y)}}{2 * C}$$

Y= the absorbency at 492 nm of samples

A, B and C are constant values calculated by the GraphPad Prism programme.

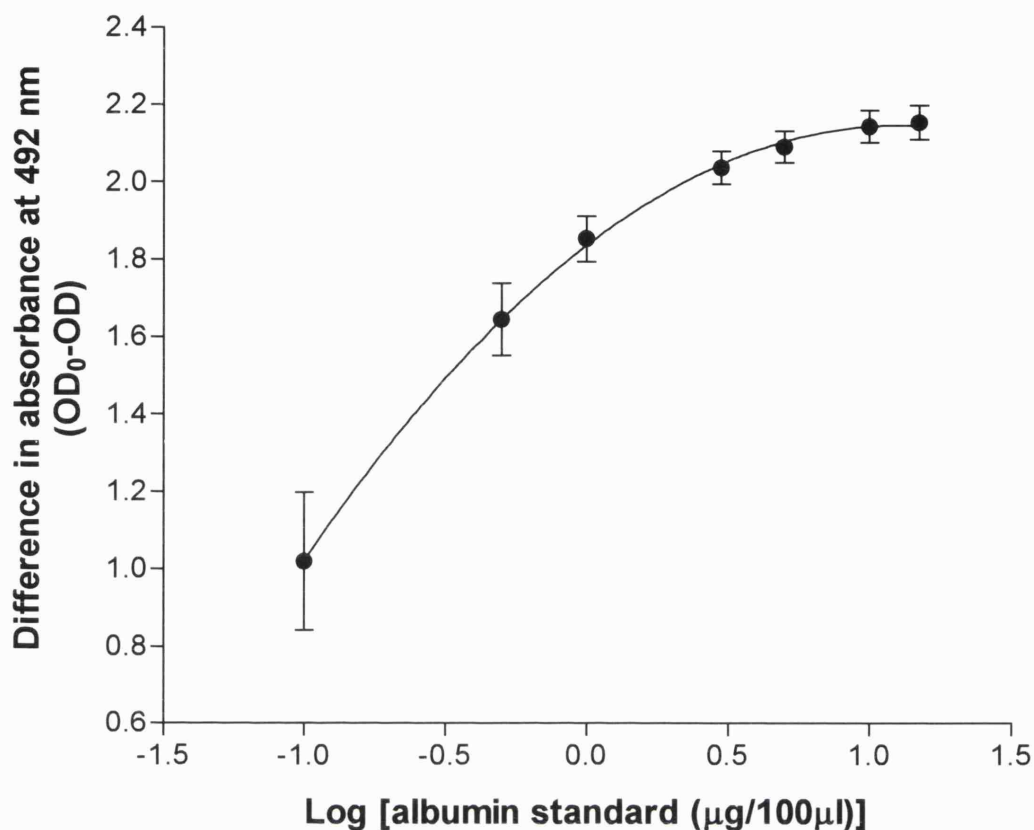


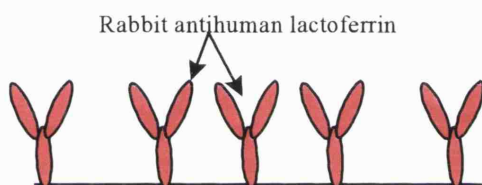
Figure 2.7: A typical standard curve for albumin ELISA, $n=5$. The data are means \pm S.E.M.

2.3.2 LACTOFERRIN MEASUREMENT

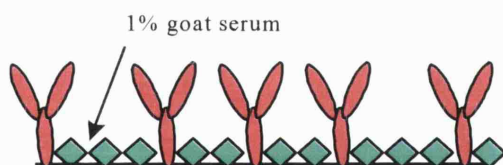
Lactoferrin was measured by direct sandwich ELISA (Raphael *et al.*, 1989a) in which the antibody against lactoferrin is passively adsorbed onto a microtitre plates, and, after incubation, unadsorbed antibody is washed away. Added standard lactoferrin, or sample with an unknown lactoferrin concentration is then bound to the antibody. The bound lactoferrin is then detected by the addition of enzyme labelled antibody specific to the lactoferrin. The amount of lactoferrin in the samples was well above the detection limits. Thus prior to an assay the samples were diluted in PT (0.05% Tween 80 in PBS, pH 7.4). The samples collected after methacholine and histamine provocations were diluted 1:1000 in PT. The samples collected after bradykinin and substance P provocation were diluted 1:500 in PT. Two replicates of the lactoferrin standards and each sample were assayed. The intra- and inter-assay coefficients of

variation were 20% and 29%, respectively. The full detail of the assay is described in figure 2.8.

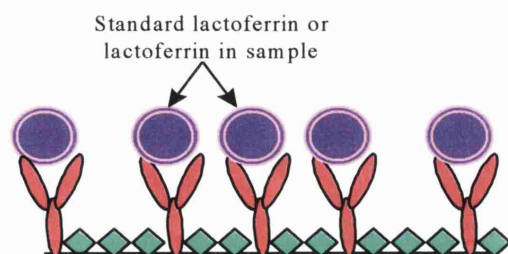
The absorbency at 492 nm for each well containing standard lactoferrin and blank was used to construct a standard curve. The absorbencies of standard lactoferrin were plotted against logarithmic values of the standard lactoferrin concentration. As in the albumin standard curve, the lactoferrin standard curve was fitted with a second order polynomial equation by the use of GraphPad Prism software. Figure 2.9 shows a typical lactoferrin standard curve. The value of lactoferrin in samples can be obtained by the same procedure as in albumin assay.



Stage (i): 100 μ l of rabbit antihuman lactoferrin diluted 1:1000 in 0.1M carbonate buffer (pH 9.6) was added to each well of a microtiter plate. The plate was incubated at 37 °C for 90 minutes.



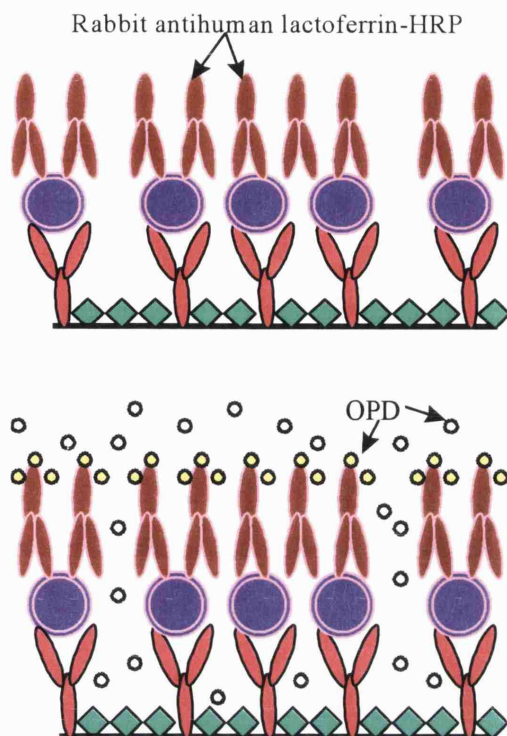
Stage (ii): The plate was then washed 4 times with PT (0.05% Tween 80 in PBS pH 7.4).



Stage (iii): The wells were blocked with 1% goat serum (200 μ l per well) diluted in PT.

Stage (iv): After incubation with 1% goat serum the plate was washed 4 times with PT.

Stage (v): 100 μ l of standard lactoferrin or samples diluted 1:1000 or 1:500 in PT were then incubated at 37 °C for 90 minutes.



Stage (vi): The wells were again washed 4 times. 100 μ l of diluted (1:1000 in PT) enzyme labelled antibody, rabbit antihuman lactoferrin-HRP, was added to each well and again incubated 37 °C for 90 minutes.

Stage (vii): After the plate was washed, the reaction was developed with OPD, and then stopped with 1M H_2SO_4 and the plate was read at 492 nm.

Figure 2.8: Direct sandwich ELISA for lactoferrin.

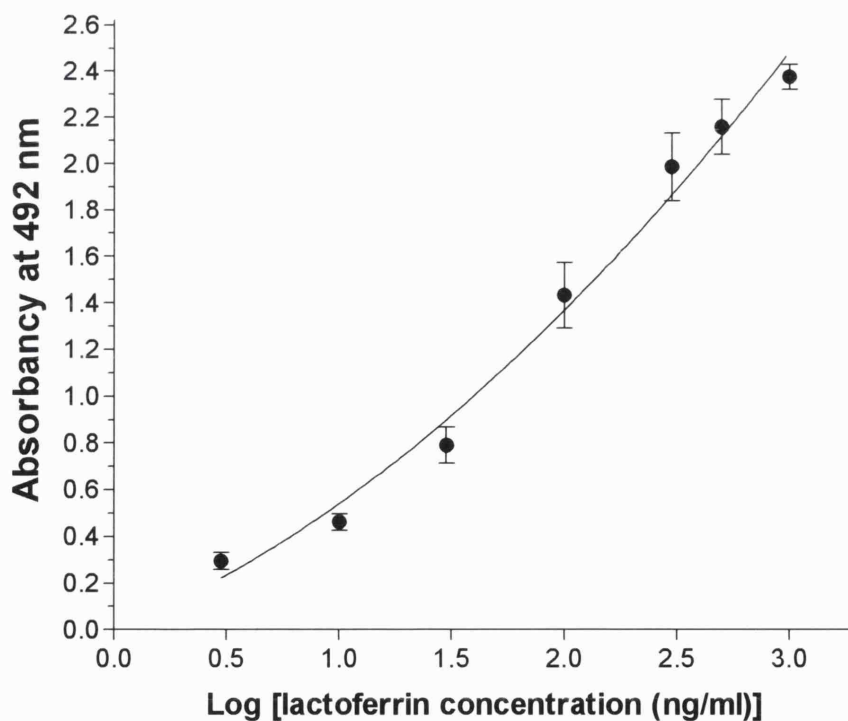


Figure 2.9: A typical standard curve for lactoferrin ELISA, $n=5$. The data are means \pm S.E.M.

2.3.3 LYSOZYME MEASUREMENT

The measurement of lysozyme concentration was carried out by a turbidimetric assay. The assay was based on the enzymatic hydrolysis of bacterial cell walls. 1 ml of 0.25mg/ml, in 0.1 M PBS (pH 7.0), *Micrococcus lysodeikticus* was added to a cuvette and the absorbency at 450 nm (t_0) was recorded. 50 μ l of lysozyme standard or undiluted sample was then added to the 1 ml suspension of *Micrococcus lysodeikticus*. Six minutes after the addition of lysozyme standard or sample the absorbency at 450 nm (t_6) was recorded. Two replicates of the lysozyme standards and each undiluted sample were assayed. The intra- and inter-assay coefficients of variation were 15% and 24%, respectively. A plot of the difference in the absorbency before, and after, the addition of lysozyme standard or sample (t_0-t_6), versus concentration of standard lysozyme was constructed as a standard curve (figure 2.10). The line of best fit was calculated, again by using GraphPad Prism software, with linear regression where the equation for a straight line ($Y = m \cdot X + C$) is applied. The value of lysozyme in the samples can then be read directly from the graph or solving the straight line equation:

$$X = \frac{Y - C}{m}$$

Y is the difference in the absorbance before and after addition of lysozyme standard or sample (t_0-t_6), C is a constant value at the y-axis intersection and m is the gradient of the line.

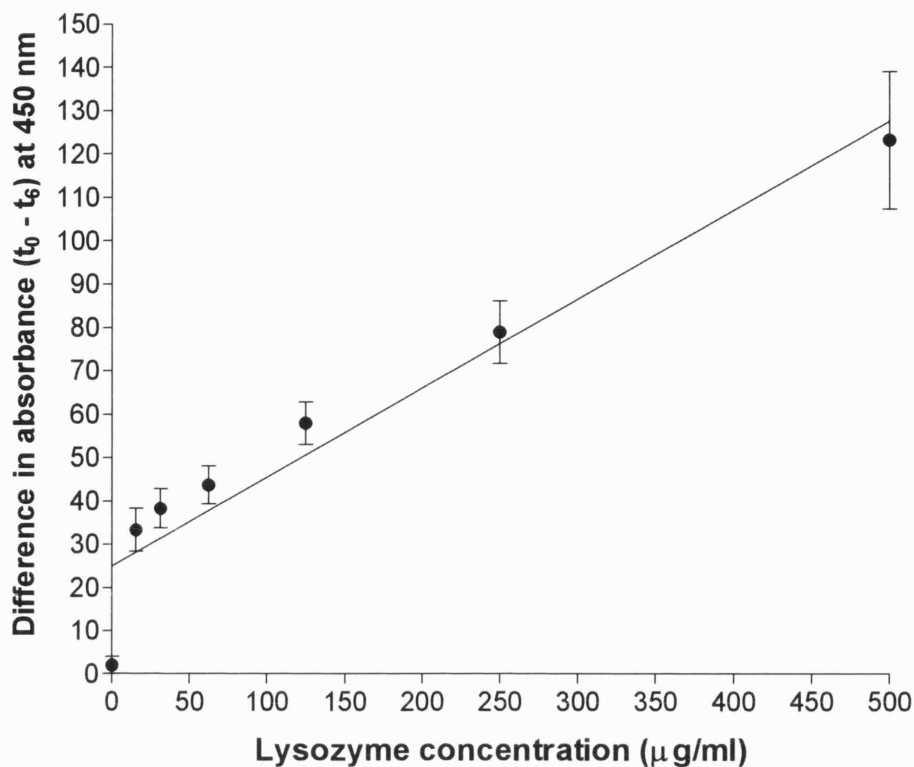


Figure 2.10: A typical lysozyme standard curve, $n=5$. The data are means \pm S.E.M.

2.3.4 MUCOUS GLYCOPROTEIN (MGP) MEASUREMENT

Mucous glycoprotein is a macromolecule secreted from submucosal gland mucous cells and goblet cells. MGP consists of a protein core onto which oligosaccharide side chains are attached. The oligosaccharide side chains are attached to the peptide region by O-glycosidic linkages between N-acetylglucosamine and threonine or serine (figure 2.11). These oligosaccharide side chains contain five sugars: fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid. Fucose and N-acetylneuraminic acid always occupy terminal (unsubstituted) portions. The peptide portion of mucous glycoprotein contains 40% or more hydroxyaminoacids (threonine and serine) and as much as 70% hydroxyaminoacids plus proline, glycine and alanine.

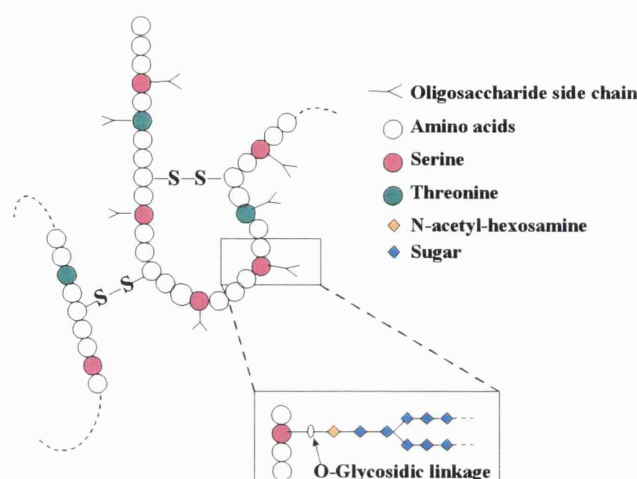


Figure 2.11: Structure of mucous glycoprotein (Kalinin *et al.*, 1986).

Quantitation of MGP by a sandwich ELISA method has been described by Mullol *et al.* (1992c). However the antibodies needed for such an assay are not available commercially, therefore, other techniques have to be taken into consideration. Fucose is a terminal sugar specific to mucous glycoproteins and is virtually absent from serum glycoproteins (Lopez-Vidriero & Reid, 1978). Therefore, many studies have used fucose as a marker of MGP (Brofeldt *et al.*, 1986; Baraniuk *et al.*, 1992b).

The method of fucose analysis, which is used as an estimation of MGP in this study, has been described by Winzler (1955). After the lavage was centrifuged and the supernatant was separated for further analysis; 2 ml of the pellet, containing gel-like secretion, was precipitated by 95% ethanol. After centrifugation and a second precipitation by ethanol, the precipitate was dissolved in 2 ml 0.1 M NaOH. The suspension was then divided equally into two boiling tubes, 4 ml of ice cold $\text{H}_2\text{SO}_4\text{-H}_2\text{O}$ mixture (6:1) was added to each tube. The tubes were heated in a boiling water bath for 3 minutes. After the tubes were cooled down, 0.1 ml cysteine reagent was added to one of the tubes, but not to the other, as this was used to correct for non-specific colour development. After 90 minutes the absorbance at 396 nm was measured. The fucose concentration in samples was interpolated from a standard curve which, as previously described in section 2.3.1, was constructed by plotting the

difference between absorbency of sample with cysteine reagents and that without ($OD_{(+)} - OD_{(-)}$) against the logarithmic value of the standard fucose concentration. The intra- and inter-assay coefficients of variation were 23% and 50%, respectively. As with albumin and lactoferrin the curve was fitted with the second order polynomial equation (figure 2.12). The logarithmic value of the fucose concentration can then be obtained by either direct reading from the standard curve or solving the second order polynomial equation as already described.

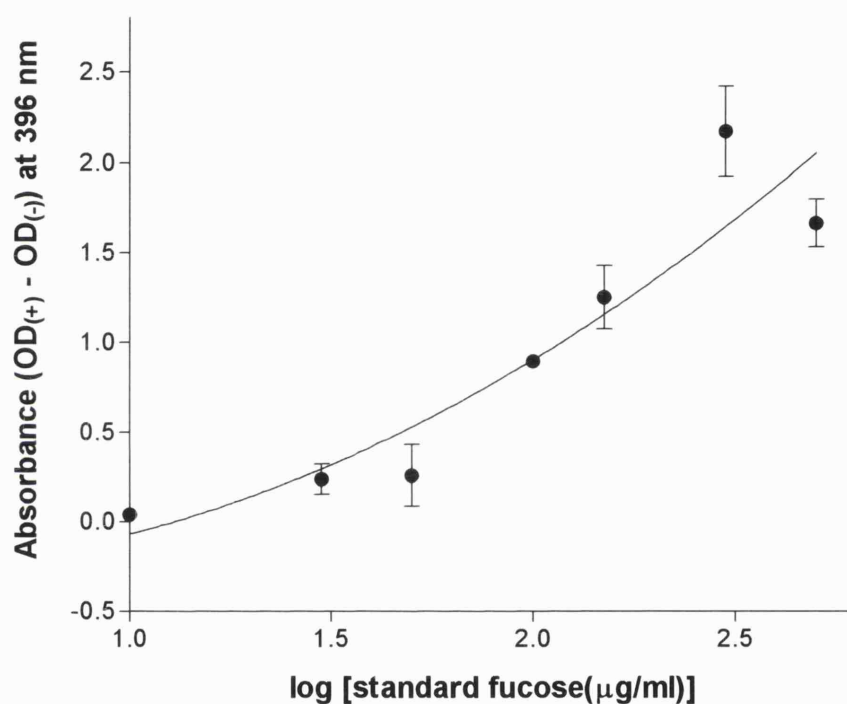


Figure 2.12: A typical fucose standard curve, $n=5$. The data are means \pm S.E.M.

CHAPTER 3

THE EFFECT OF METHACHOLINE, HISTAMINE AND BRADYKININ ON NASAL SECRETION AND THE PROTEIN CONTENT IN NASAL LAVAGE.

3.1 Introduction

The experiments in this chapter were conducted to study the effect of three substances, methacholine, histamine and bradykinin, on the secretory response of the human nasal mucosa. The nasal mucosa is richly innervated by the parasympathetic nervous system. The parasympathetic nervous system plays an important role in nasal responses to external stimuli. The parasympathetic discharge will release acetylcholine and neuropeptides, and will result in glandular discharge and vasodilatation. The release of acetylcholine is paramount in controlling submucosal glandular secretion in the nasal mucosa (Konno & Togawa, 1979). Methacholine, an analogue of acetylcholine, has been used to demonstrate the cholinergic reactivity of the nasal mucosa. Methacholine stimulated secretion from submucosal glands both *in vitro* (Patow *et al.*, 1984) and *in vivo* (Devillier *et al.*, 1988; Raphael *et al.*, 1988; Gawin *et al.*, 1991). In this chapter, methacholine was used as an indicator of the secretory response and to further investigate the use of the cotton buds as a tool to assess the change in the amount of nasal secretion. In addition, the source of proteins in methacholine-induced nasal secretion was studied.

Since its discovery, histamine has been recognised as a major mediator of allergic reactions and diseases. It has been found in nasal lavage following allergen challenge of allergic subjects (Naclerio *et al.*, 1983a; Naclerio *et al.*, 1983b; Norman *et al.*, 1985; Wagenman *et al.*, 1997). A number of studies have documented that intranasal challenge with histamine resulted in nasal obstruction, measured by an increase in nasal airway resistance (McClean *et al.*, 1977; Britton *et al.*, 1978; Doyle *et al.*, 1990;

Braunstein *et al.*, 1992; Hilberg *et al.*, 1995b; Austin & Foreman, 1994b). The effect of antihistamines on histamine-induced and allergen-induced nasal responses has been widely examined. Antihistamines have been shown to reduce an increase in nasal airway resistance induced by histamine (Braunstein *et al.*, 1992; Hilberg *et al.*, 1995b; Frossard *et al.*, 1997). Antihistamines are widely used in the treatment of allergic rhinitis further implicating the importance of histamine as a mediator of rhinitis. In this chapter, the effect of histamine on nasal secretory activity, and the source of proteins in nasal secretion induced by histamine challenges were investigated.

Bradykinin is another mediator that has been proposed as a potentially important mediator of allergic rhinitis. Bradykinin is generated during the pathology of allergic rhinitis (Dolvovich *et al.*, 1970), and following experimentally induced allergic reactions in rhinitic subjects (Proud *et al.*, 1983 and 1986; Norman *et al.*, 1985). The generation of bradykinin during both the early- and late-phase allergic reactions correlated with the onset of clinical symptoms (Naclerio *et al.*, 1985a; Proud *et al.*, 1986a, 1986b and 1989). Nasal challenge with bradykinin leads to an increase in nasal airway resistance (Doyle *et al.*, 1990; Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a and 1994b) and an increase in vascular permeability (Proud *et al.*, 1988; Baranuik *et al.*, 1994b; Holmberg *et al.*, 1990). The effect of nasal challenge with bradykinin on nasal secretory activity and the source of proteins in bradykinin-induced nasal secretion were examined in this chapter.

The aim of this study was, therefore, to investigate the effect of methacholine, histamine and bradykinin, on the secretory activity of the nasal mucosa and on the source of proteins following nasal challenges.

3.2 Experimental protocol

For the following experiments, the study of nasal secretory activity was conducted on separate occasions from the study of the proteins released. Normal, healthy volunteers aged 20 to 40 years were used. No subject was taking any medication at the time of the experiments and in the week prior to the study. A double-blind, cross-over design was used in all of the protocols.

3.2.1 Effect of methacholine on nasal secretory activity and the release of albumin, lactoferrin, lysozyme and mucous glycoprotein into the nasal cavity of normal subjects.

The baseline nasal secretion was obtained prior to nasal challenge with methacholine. Three minutes after the baseline measurement, methacholine was delivered into the nasal cavity by a handheld pump spray. The first measurement of nasal secretion following methacholine challenge was then taken 1 minute after the challenge with 1 minute allowed for absorption of secretion into the cotton bud. Then further measurements were taken similarly every 3 minutes. The sequence of the protocol is illustrated in Figure 3.1

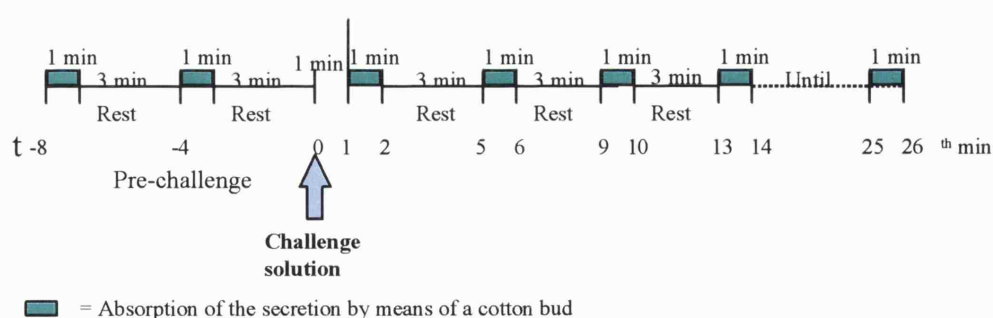


Figure 3.1: Protocol for investigating the effect of methacholine, histamine and bradykinin on the nasal secretion response.

In the study of the protein release, nasal lavage was performed three times per nostril, prior to nasal challenge. The third lavage was used for the measurement of baseline protein content in the lavage. Then methacholine was delivered into the nasal cavity as in the secretory study. A nasal lavage was performed 10 minutes after the challenge. The concentration of lactoferrin, lysozyme, albumin and mucous glycoprotein (measured as fucose) in the lavage was determined by the methods, which have already been described in chapter 2.

The doses of methacholine used for both studies were 1, 3 and 10 mg. These chosen doses were within the range used in previous studies (Devillier *et al.*, 1988; Raphael *et al.*, 1988). Each subject received the treatment on separate days, at least 24 hours apart. Each subject received all of the doses including saline as a control. The order of the doses was determined randomly.

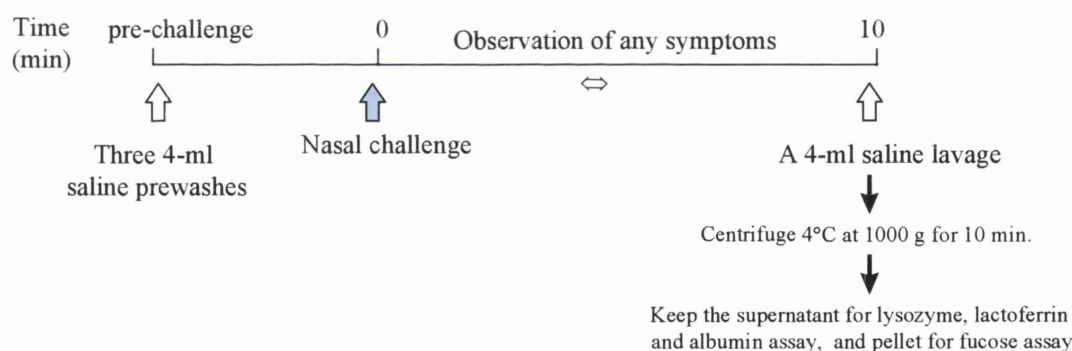


Figure 3.2 Protocol for investigating the effect of methacholine, histamine and bradykinin on the concentration of lactoferrin, lysozyme, albumin and mucous glycoprotein (MGP) recovered after the nasal challenge.

3.2.2 Effect of histamine on nasal secretory activity and the release of albumin, lactoferrin, lysozyme and MGP into the nasal cavity of normal subjects.

The protocol was similar to that for the methacholine study, however 100, 300 and 1000 µg of histamine were used in these studies. These doses have previously been

shown to induce an increase in NAR (Austin *et al.*, 1994b). Again, each subject received all of the doses and saline on separate occasions, at least 24 hours apart and the order of the treatments was determined randomly.

3.2.3 Effect of bradykinin on nasal secretory activity and the release of albumin, lactoferrin, lysozyme and MGP into the nasal cavity of normal subjects.

The same protocol as for methacholine and histamine studies was employed in the study of the bradykinin effect. 30, 100 and 300 µg per nostril were the doses used in the studies. These doses have previously been shown to induce an increase in NAR (Austin *et al.*, 1994b). Again, each subject received all of the doses and saline on separate occasions, at least 24 hours apart and the order of the dosage was determined randomly.

3.2.4 Record of symptoms

The number of sneezes was counted and recorded after every nasal challenge. Any other symptoms, such as itching, pain and sore throat, experienced were given voluntarily by the subjects and noted.

3.2.5 Data analysis

The medians and percentiles were determined for all parameters; rate of nasal secretion (mg/min), area under the curve (AUC) of secretion response over 26 minutes observation and protein concentration in nasal lavage. Friedman's test was performed to compare the effects of a series of increasing doses of challenge solution on a group of individuals. Dunn's test was performed following the Friedman's test, when there was a statistically significant difference in the treatments, in order to determine exactly which treatment had an effect in comparison to saline treatment. This test was

performed on the secretory response at each time point, on the AUC and on protein content in nasal lavage. A $p\text{-value} \leq 0.05$ was considered to be significant.

3.3 Results

3.3.1 (i) *Effect of methacholine on nasal secretion in normal subjects.*

Application of 1, 3 and 10 mg methacholine induced a nasal sensation of warmth and a throbbing sensation that was reported by all subjects. Methacholine 10 mg also produced facial flush in 6 of 8 individuals. No other symptoms were observed. Control saline challenge produced no nasal symptoms. Methacholine 3 and 10 mg induced nasal secretion within 2 minutes following its application (Friedman test $p\text{-value} < 0.001$, Dunn's test $p\text{-values} < 0.05$ for both doses in comparison to saline, at $t = 2$ minutes). The secretory response reached maximal intensity within 6 minutes after the challenge of 10 mg methacholine (Friedman test $p\text{-value} < 0.001$, Dunn's test $p\text{-values} < 0.05$ when comparison with saline, at $t = 6$ minutes). The duration of effect of methacholine on nasal secretion, expressed as median rate is presented in Figure 3.3 (A). The effect of 10 mg methacholine did not return to the baseline until after 18 minutes.

Figure 3.3 (B) shows that methacholine induced an increase in nasal secretion (Friedman's test, $n=8$, $p\text{-value} < 0.01$) with a significant increase in response after 3 and 10 mg of methacholine over that of saline (Dunn's test, $p\text{-values} < 0.05$, $n=8$). The response appeared to be dose-related, however there were no statistically significant difference between the responses induced by each dose of methacholine.

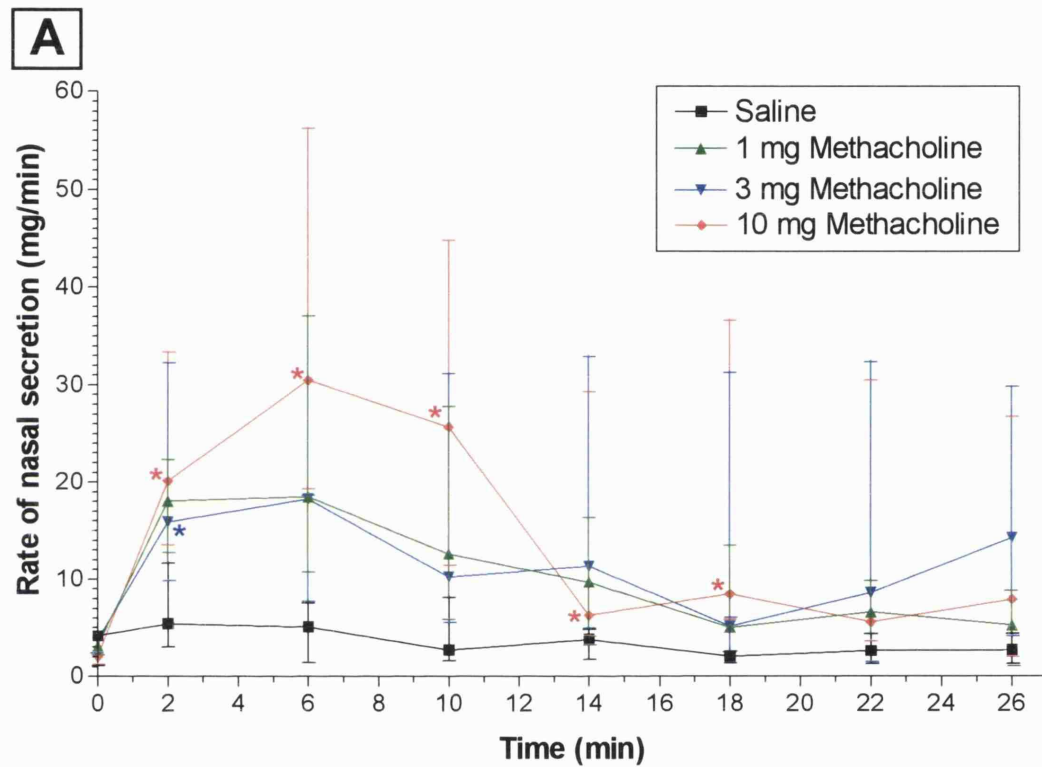


Figure 3.3 (A): The effect of methacholine on the secretory response. The data are the medians of 8 subjects, with the 25th and 75th percentile values. The graph shows the duration of effect of methacholine over 26 minutes. A statistically significant difference from the saline value at each time point is indicated by * (* p-value < 0.05).

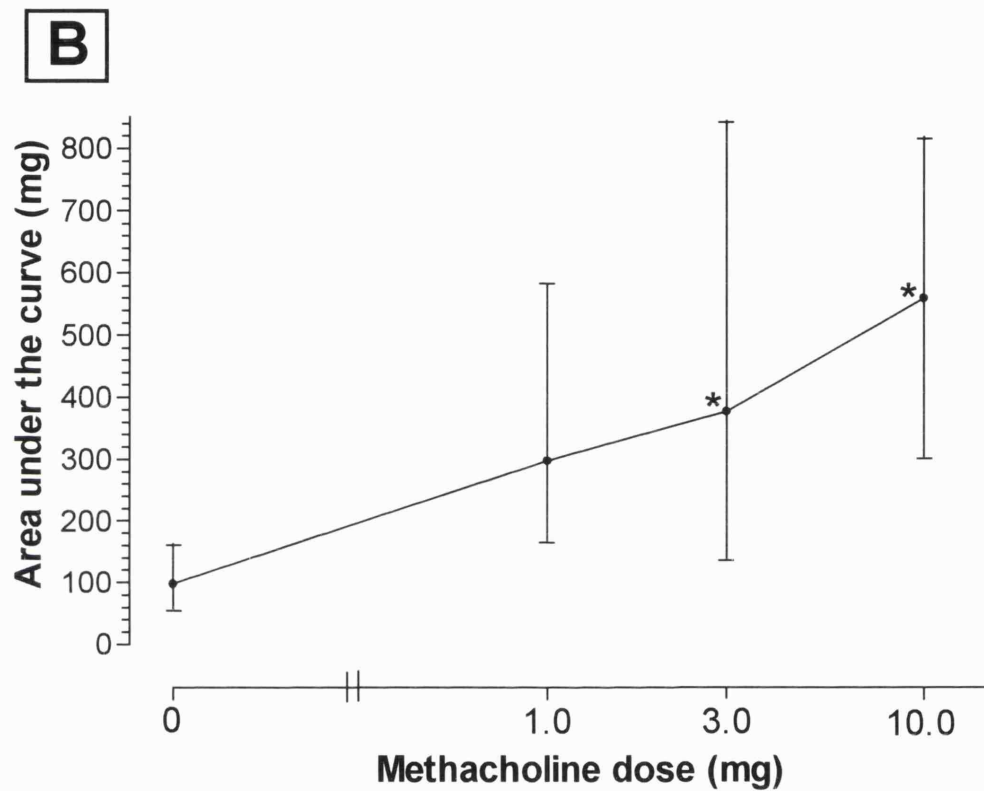


Figure 3.3 (B): Representation of the dose-response relationship between methacholine dose, plotted on a \log_{10} scale, and the secretory response over 26 minutes. The data are the medians of 8 subjects, with the 25th and 75th percentile values. There was a significant nasal response to methacholine, which was evaluated by the Friedman's test (p -value < 0.01). * indicates a statistically significant difference from the saline value (* p -value < 0.05).

3.3.1 (ii) Effect of methacholine on the release of albumin, lactoferrin, lysozyme and mucous glycoprotein (measured as fucose) into the nasal cavity of normal subjects.

Seven subjects took part in the methacholine-induced proteins secretion study. In all subjects, at 3 and 10 mg of methacholine, watery secretion began to drip from the nose within 2 minutes of the challenge. The dripping secretion was collected into a test tube via a funnel, which was placed in the test tube. The same test tube was then used to collect the final lavage 10 minutes after the challenge.

Methacholine induced a significant increase in lactoferrin, lysozyme, albumin and mucous glycoprotein in the nasal lavage (figure 3.4 (A) p -value < 0.001 ; p -value < 0.05 figure 3.4 (B); figure 3.4 (C) and (D) p -values ≤ 0.01 , analysed by Friedman's test). In comparison with protein concentration in the nasal lavage after saline challenge, 10 mg of methacholine induced a significant increase in the concentration of all measured proteins (Dunn's test, p -values < 0.05 for lactoferrin, lysozyme, albumin and fucose). In addition to significant responses following 10 mg of methacholine, 3 mg of methacholine also induced a significant increase in lactoferrin and lysozyme concentrations (Dunn's test, p -values < 0.05).

Lysozyme, albumin and fucose responses appeared to be dose-related, however there were no statistically significant difference between the responses induced by each dose of methacholine (Dunn's test, p -values > 0.05). Lactoferrin response on the other hand appeared to be dose-related; 10 mg methacholine induced a significantly higher lactoferrin in the nasal lavage than those induced by 1 mg methacholine (Dunn's test, p -value < 0.05).

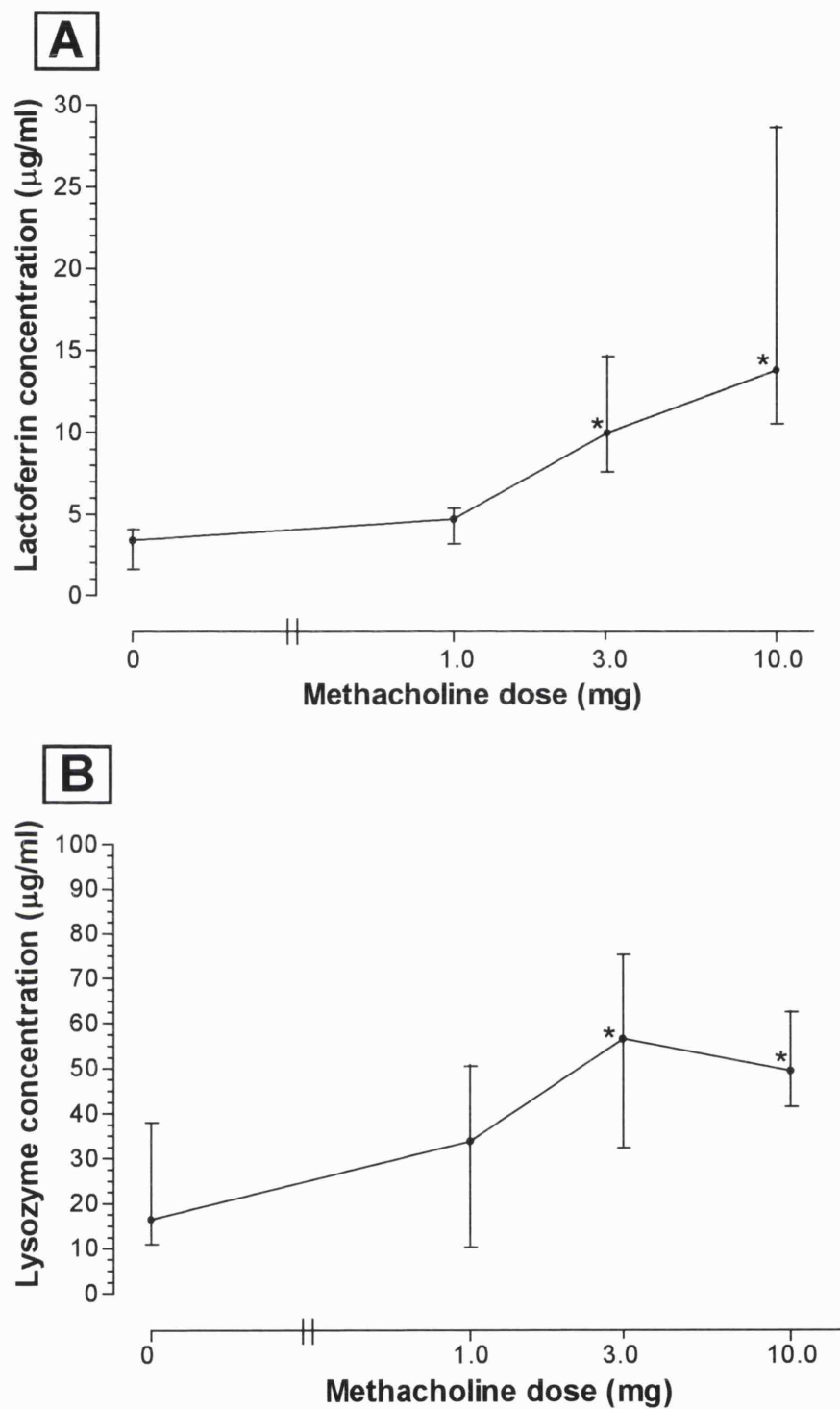


Figure 3.4 (A) and (B): Dose-response of lactoferrin (A) and lysozyme (B) concentration in nasal lavage following increasing doses of methacholine. The data are the medians value of 7 subjects, with the 25th and 75th percentile values. The dose of methacholine was plotted on a \log_{10} scale against the concentration of protein in nasal lavage. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05).

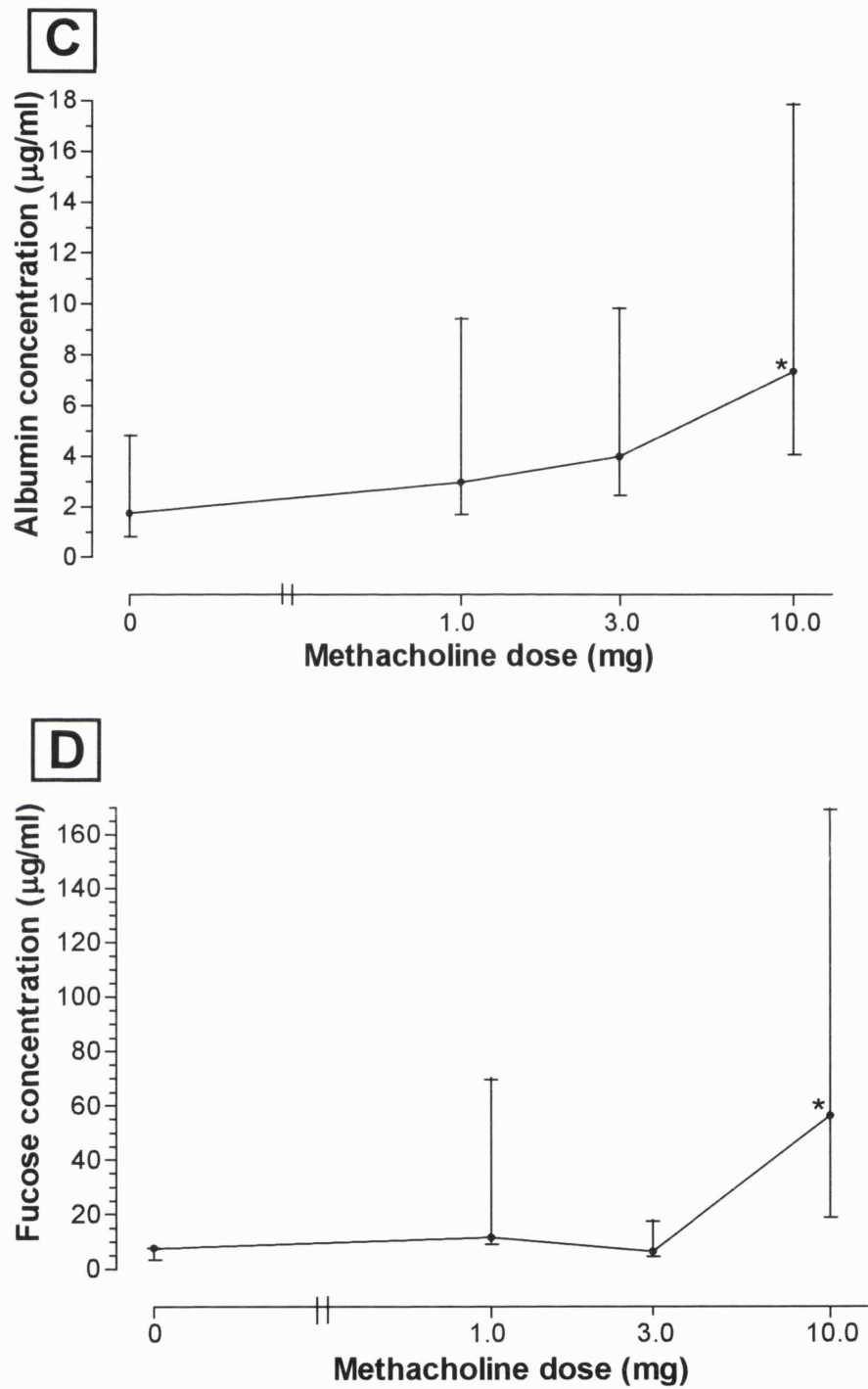


Figure 3.4 (C) and (D): Dose-response of albumin (C) and fucose (D) concentration in nasal lavage following increasing doses of methacholine. The data are the medians value of 7 subjects, with the 25th and 75th percentile values. The dose of methacholine was plotted on a log₁₀ scale against the concentration of protein in nasal lavage. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05).

3.3.2 (i) Effect of histamine on nasal secretion in normal subjects.

Figure 3.5 (A) shows the duration of the effect of histamine and saline on nasal secretion. Seven subjects were given a nasal challenge with saline, and histamine at doses of 100, 300 and 1000 μg in each nostril. Saline provocation produced a relatively constant level of nasal secretion except at 2 minutes where the amount of secretion collected appeared to be increased, but this could be the volume of challenge. The saline challenge produced no clinical symptoms but histamine challenges, in contrast, produced sneezing and itching in all subjects. No other symptoms were observed. Table 3.1 shows median number of sneezes following histamine provocation.

Table 3.1: The median number of sneezes following histamine challenge in 7 subjects.

Histamine dose	Median number of sneezes	25% percentile	75% percentile
100 μg	1	0	2
300 μg	2	0	4
1000 μg	3	2	8

Histamine induced a significant increase in nasal secretion within 2 minutes following its application (Friedman's test, $p\text{-value} < 0.001$ at $t=2$ minutes). When a statistical comparison by Dunn's test was employed, the lowest dose of histamine failed to produce a significant increase in secretion with respect to saline ($p\text{-values} > 0.05$ for the response at each time point, $n=7$). Histamine 300 and 1000 μg , on the other hand, induced a significant increase in the secretory response when compared to that of saline (Dunn's test, $p\text{-values} < 0.05$, $n=7$, for both doses and at 2 minutes and 6 minutes and for 1000 μg at 10 minutes and 14 minutes), figure 3.5 (A).

Histamine caused an increase in nasal secretion, figure 3.5 (B) (Friedman's test, p -value < 0.01 , $n=7$). Histamine 300 and 1000 μg induced a significant difference in the secretion in comparison to that of saline (p -values < 0.05 , $n=7$, for both doses, evaluated by Dunn's test). The secretory response appeared to be dose-related, however there were no statistically significant differences between the responses induced by each dose of histamine (Dunn's test, p -values > 0.05).

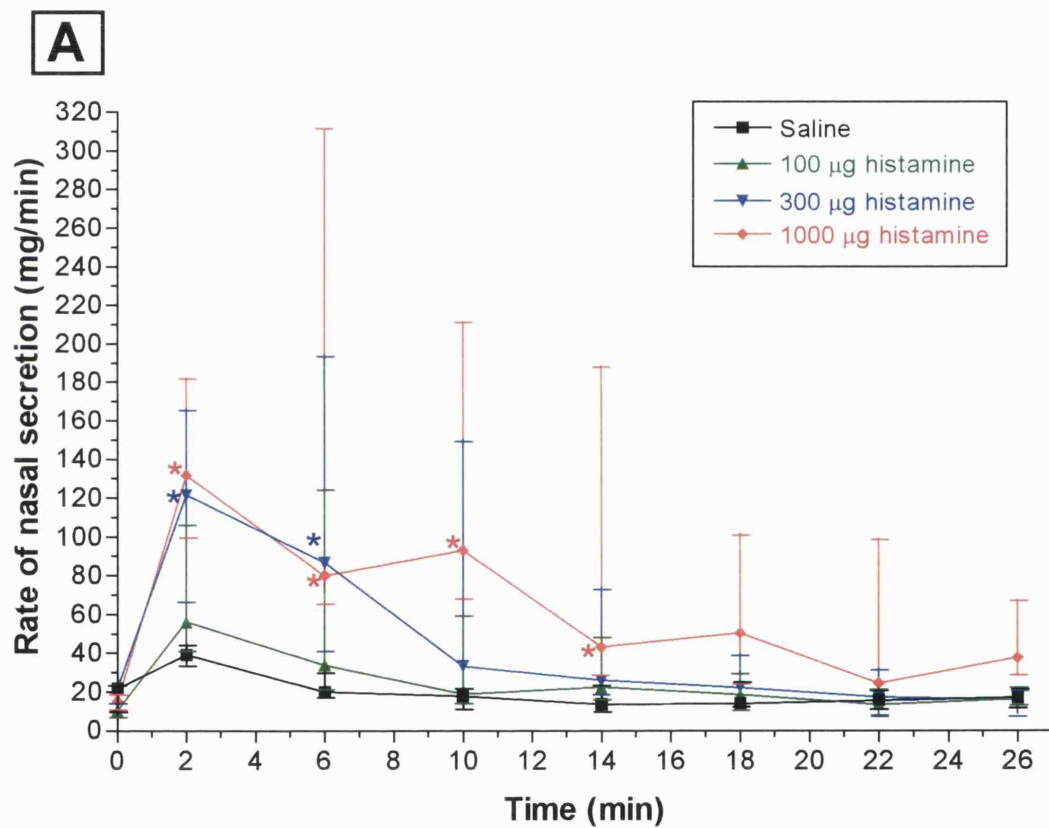


Figure 3.5 (A): The effect of histamine on the secretory response over 26 minutes. The data are the medians of 7 subjects, with the 25th and 75th percentile values. A statistically significant difference from the saline value at each time point is indicated by * (p -value < 0.05).

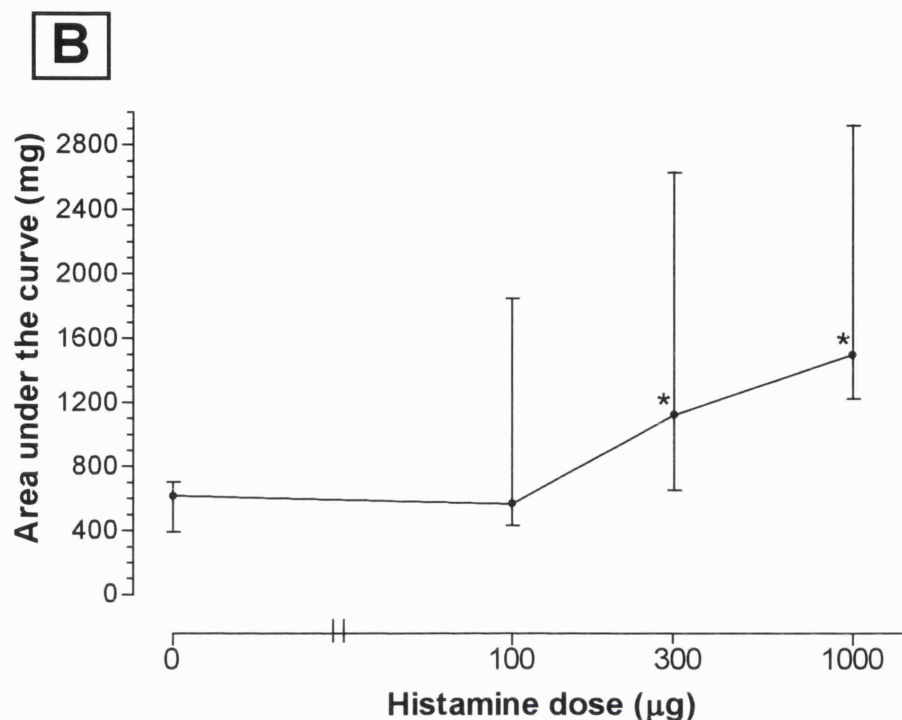


Figure 3.5 (B): Dose-response relationship between histamine dose, plotted on a \log_{10} scale, and the secretory response over 26 minutes. The data are the medians of 7 subjects, with the 25th and 75th percentile values. There was a significant secretion response to histamine, evaluated by Friedman's test (p -value < 0.01). * indicates a statistically significant difference from the saline value (* p -value < 0.05).

3.3.2 (ii) Effect of histamine on the release of albumin, lactoferrin, lysozyme and mucous glycoprotein (measured as fucose) into the nasal cavity of normal subjects.

Histamine provocation produced sneezing and itching in all subjects with the most profound symptoms occurring after the highest dose. Table 3.2 shows the median number of sneezes following histamine challenges. No other symptoms were observed. Saline provocation produced no clinical symptoms.

Table 3.2: The median number of sneezes following histamine challenge in 7 subjects.

Histamine dose	Median number of sneezes	25% percentile	75% percentile
100 µg	1	0.25	4.5
300 µg	2	0.25	8.5
1000 µg	3	2.25	10.5

Figure 3.6 shows the dose-response relationship of lactoferrin (A), lysozyme (B), albumin (C) and fucose (D) concentrations recovered by nasal lavage following nasal challenges with histamine or saline. Analysis by Friedman's test indicated that the concentration of proteins in the nasal lavage increased following histamine provocation ($n=7$; p -values < 0.05 for lysozyme and fucose, p -values < 0.01 for lactoferrin and albumin). The increase in the lactoferrin concentration following all three doses of histamine was significantly higher compared with that of saline (Dunn's test, p -values < 0.05). The increase in lysozyme concentration was significant at 300 and 1000 µg of histamine treatment in comparison to that of saline treatment (Dunn's test, p -values < 0.05). Histamine at all doses significantly increased the concentration of albumin with respect to that of saline (Dunn's test, p -values < 0.05). Histamine 100 and 1000 µg induced a significant increase in the concentration of fucose in the nasal lavage from that of saline (Dunn's test, p -values < 0.05). The lactoferrin, lysozyme and albumin responses appeared to be dose-related, however there were no statistically significant difference between the responses induced by each dose of histamine (Dunn's test, p -value > 0.05).

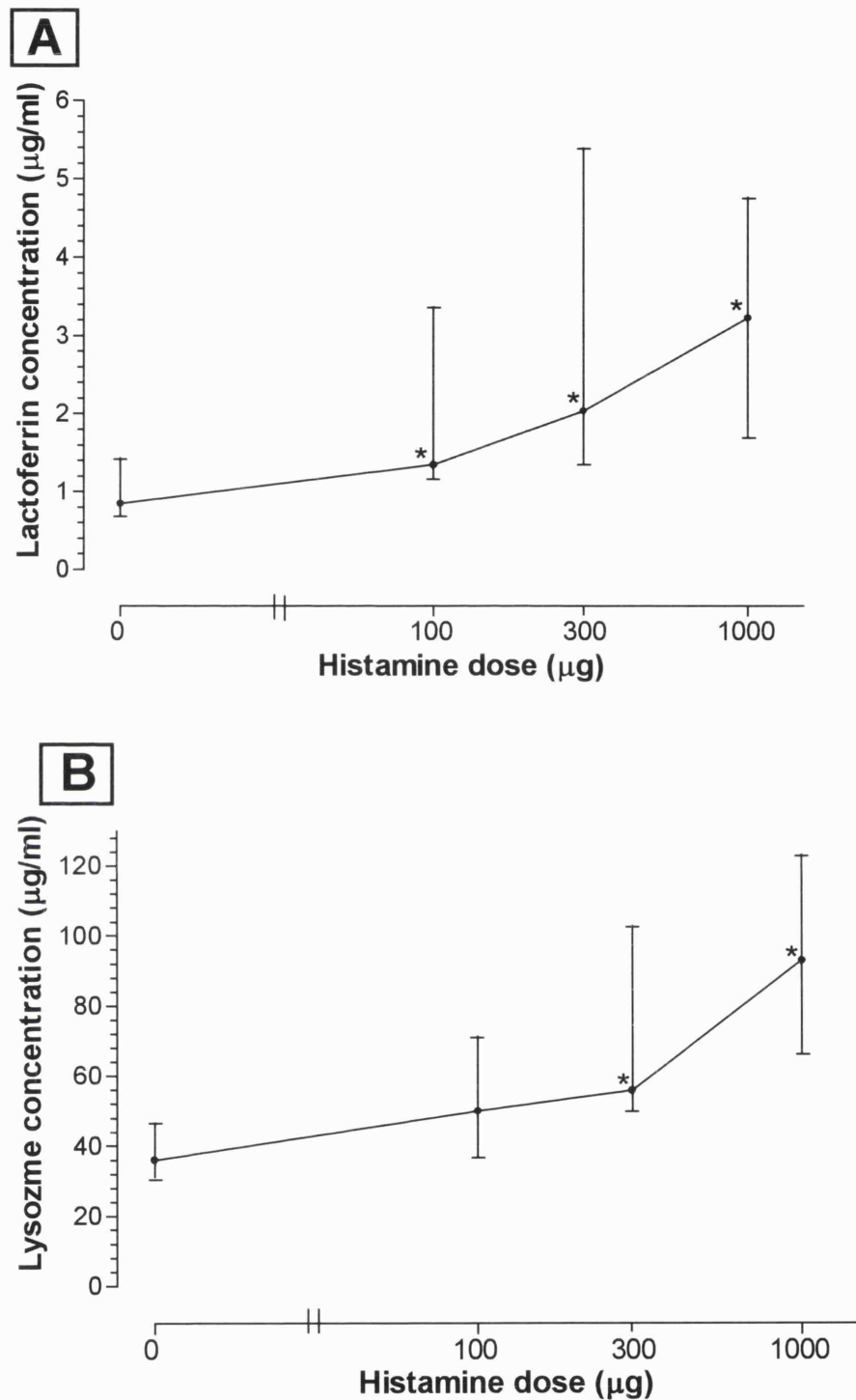


Figure 3.6 (A) and (B): Dose-response effect of histamine challenges on lactoferrin (A) and lysozyme (B) concentration in nasal lavage from 7 subjects. The dose of histamine was plotted on a \log_{10} scale against the concentration of proteins in nasal lavage expressed as the medians with the 25th and 75th percentile values. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05). The concentration of lactoferrin and lysozyme increased in response to histamine challenges (Friedman's test, p-value < 0.05).

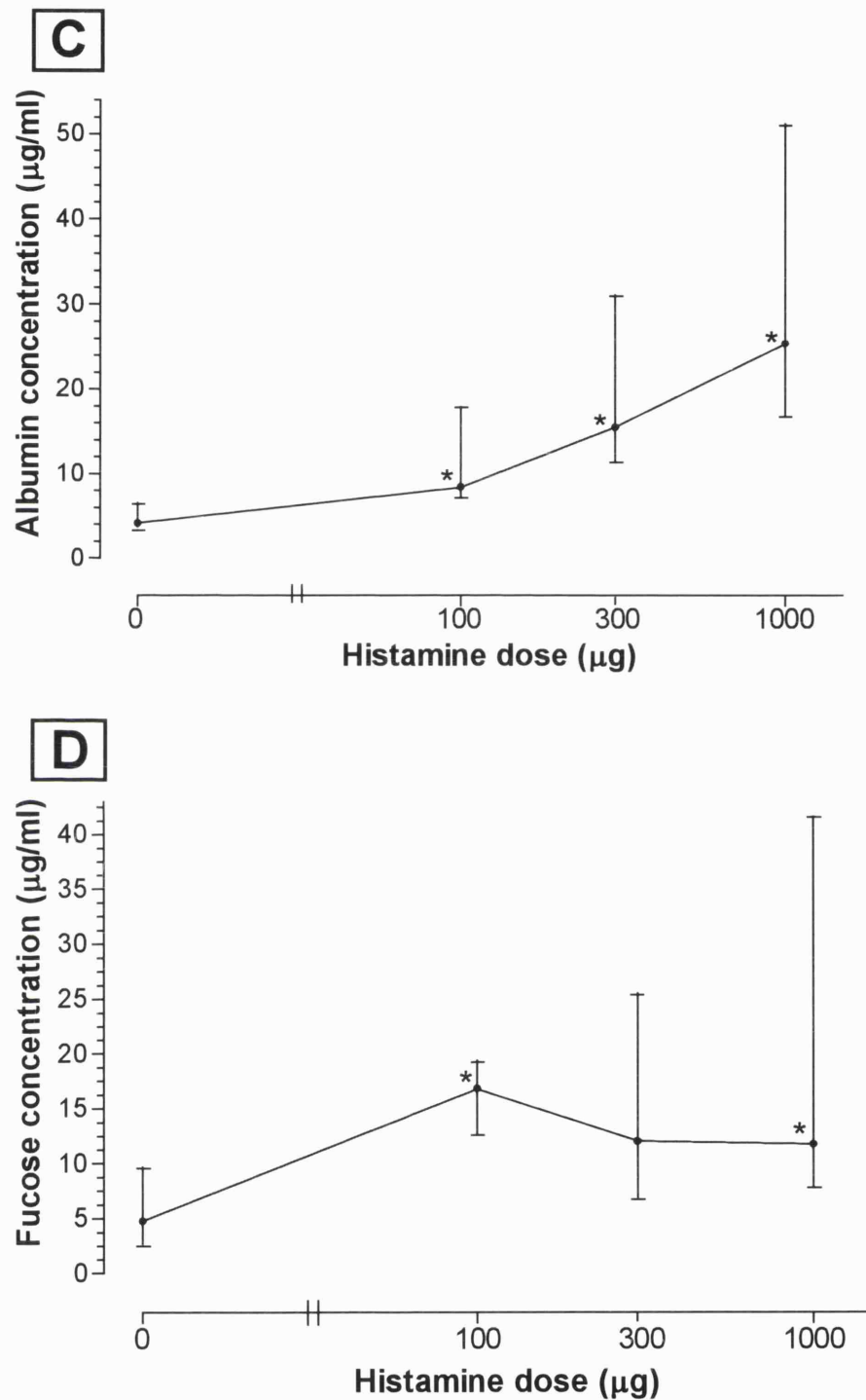


Figure 3.6 (C) and (D): Dose-response effect of histamine challenges on albumin (C) and fucose (D) concentration in nasal lavage from 7 subjects. The dose of histamine was plotted on a \log_{10} scale against the concentration of proteins in nasal lavage expressed as the medians with the 25th and 75th percentile values. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05). The concentration of albumin and fucose increased in response to histamine challenges (Friedman's test, p-values < 0.05).

3.3.3 (i) *Effect of bradykinin on nasal secretion in normal subjects.*

Ten subjects were challenged with 30, 100 and 300 µg of bradykinin and saline on separate occasions. Five subjects complained of nasal discomfort, such as sore throat and nasal pain, following 100 and 300 µg bradykinin administration. Sneezing and other symptoms were not observed. Figure 3.7 (A) shows the duration of the effect of bradykinin and saline on nasal secretion. After saline challenge the increase in the amount of secretion collected could be the result of the challenge volume. Figure 3.7 (A) shows that treatment with bradykinin induced an increase in nasal secretion within 2 minutes which lasted for 10 minutes (Friedman's test $p\text{-values} \leq 0.01$ at $t=2$ minutes and 6 minutes; $p\text{-value} < 0.02$ at $t=10$ minutes). At 2 minutes after 300 µg of bradykinin challenges, a significant increase in the nasal secretion was observed (Dunn's test, $p\text{-values} < 0.05$, with respect to that of saline). At 6 minutes after bradykinin challenges, all doses induced a significant increase in the nasal secretion in comparison to saline (Dunn's test, $p\text{-values} < 0.05$). Bradykinin at 100 and 300 µg induced a significant increase in the secretion after 10 minutes of challenge (Dunn's test, $p\text{-values} < 0.05$, with respect to that of saline). The nasal secretion returned to baseline 14 minutes after administration.

Analysis by Friedman's test indicated that bradykinin induced an increase in nasal secretion ($p\text{-value} < 0.01$, $n=10$). The increase in the secretory response over 26 minutes is significant at 100 and 300 µg of bradykinin in comparison to that of saline (Dunn's test, $p\text{-values} < 0.05$), figure 3.7 (B).

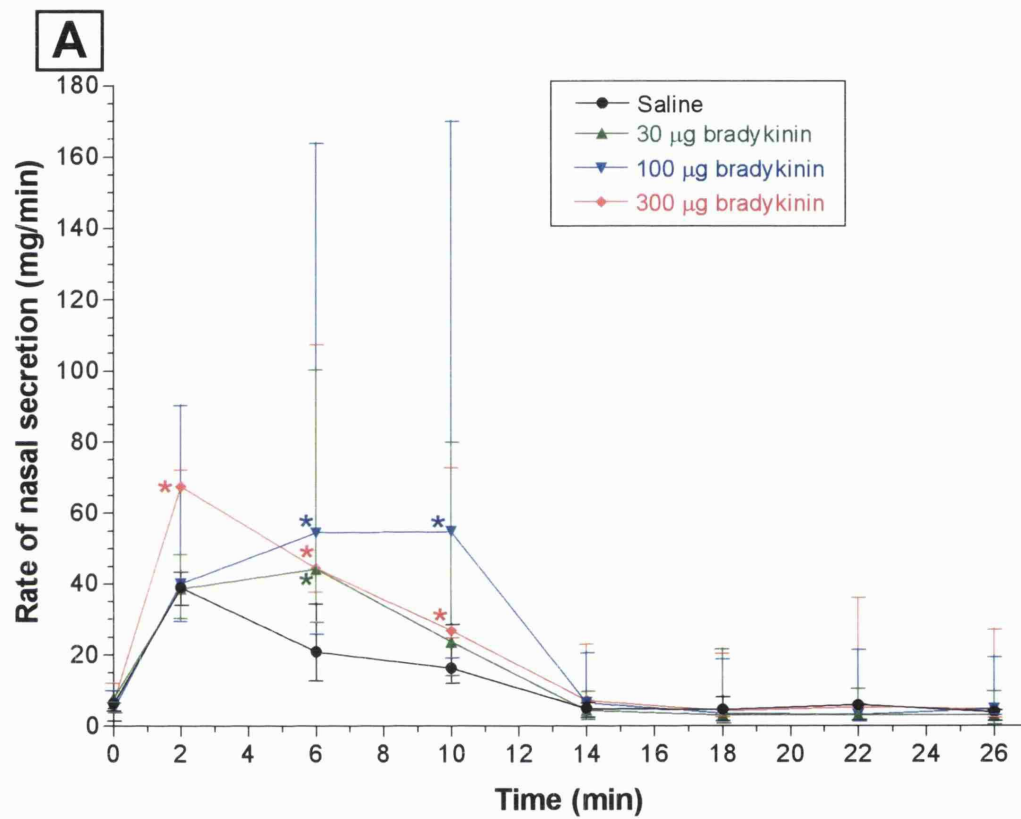


Figure 3.7 (A): The effect of bradykinin on the secretory response over 26 minutes. The data are the medians of 10 subjects, with the 25th and 75th percentile values. A statistically significant difference from the saline value at each time point is indicated by * (*p-value < 0.05).

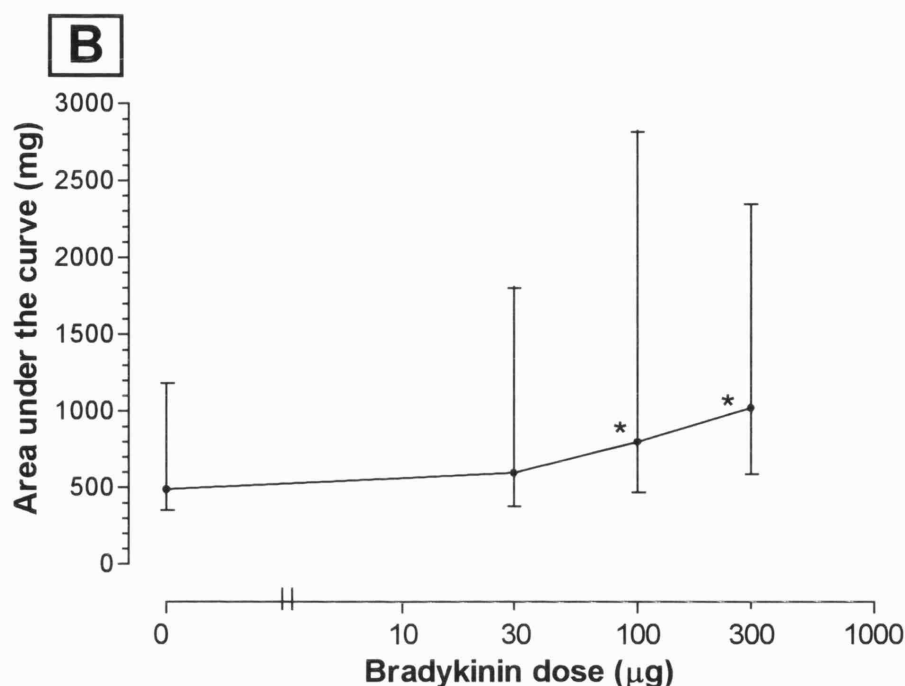


Figure 3.7 (B): Dose-response relationship between bradykinin dose, plotted on a \log_{10} scale, and the secretory response over 26 minutes. The data are the median values of 10 subjects, with the 25th and 75th percentile values. There was a significant secretory response to bradykinin challenges (Friedman's test, p -value < 0.05). A statistically significant difference from the saline value is indicated by * (* p -value < 0.05).

3.3.3 (ii) Effect of bradykinin on the release of albumin, lactoferrin, lysozyme and mucous glycoprotein (measured as fucose) into the nasal cavity of normal subjects.

The protein release response to an increasing dose of bradykinin is presented in figure 3.8. Five subjects complained of nasal discomfort, such as sore throat and nasal pain. Bradykinin produced an increase in albumin concentration in nasal lavage (Friedman's test, p -value < 0.02 , $n=8$). The increase in albumin concentration in response to bradykinin was significant at 100 and 300 μg of bradykinin (Dunn's test, $n=8$, p -values < 0.05). Bradykinin had no effect on lactoferrin, lysozyme and mucous glycoprotein release (Friedman's test, $n=8$, p -values > 0.2).

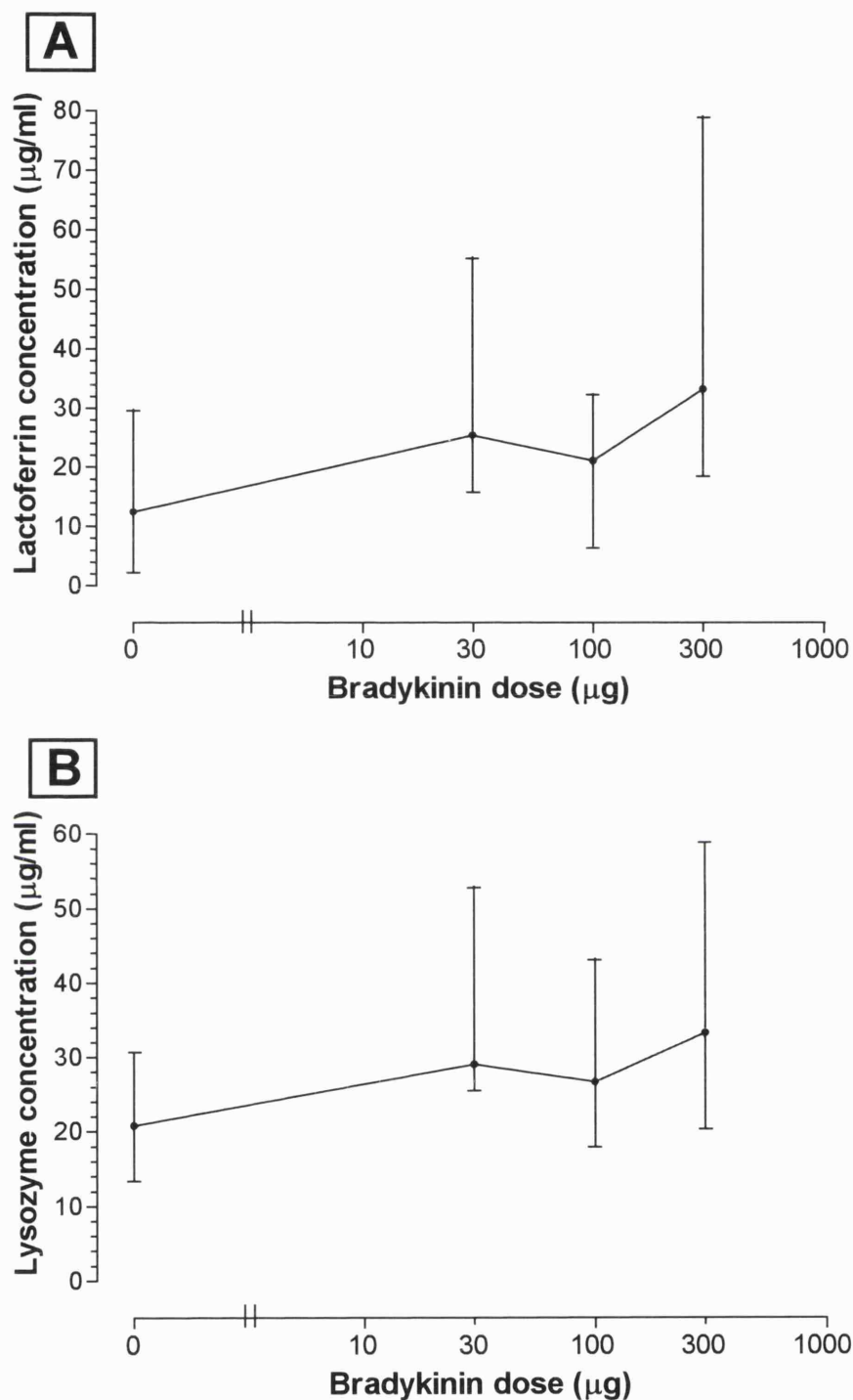


Figure 3.8 (A) and (B): Dose-response of lactoferrin (A) and lysozyme (B) concentration in nasal lavage following increasing dose of bradykinin. The data are the medians of 8 subjects, with the 25th and 75th percentile values. The dose of bradykinin was plotted on a \log_{10} scale against the concentration of proteins in nasal lavage. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05).

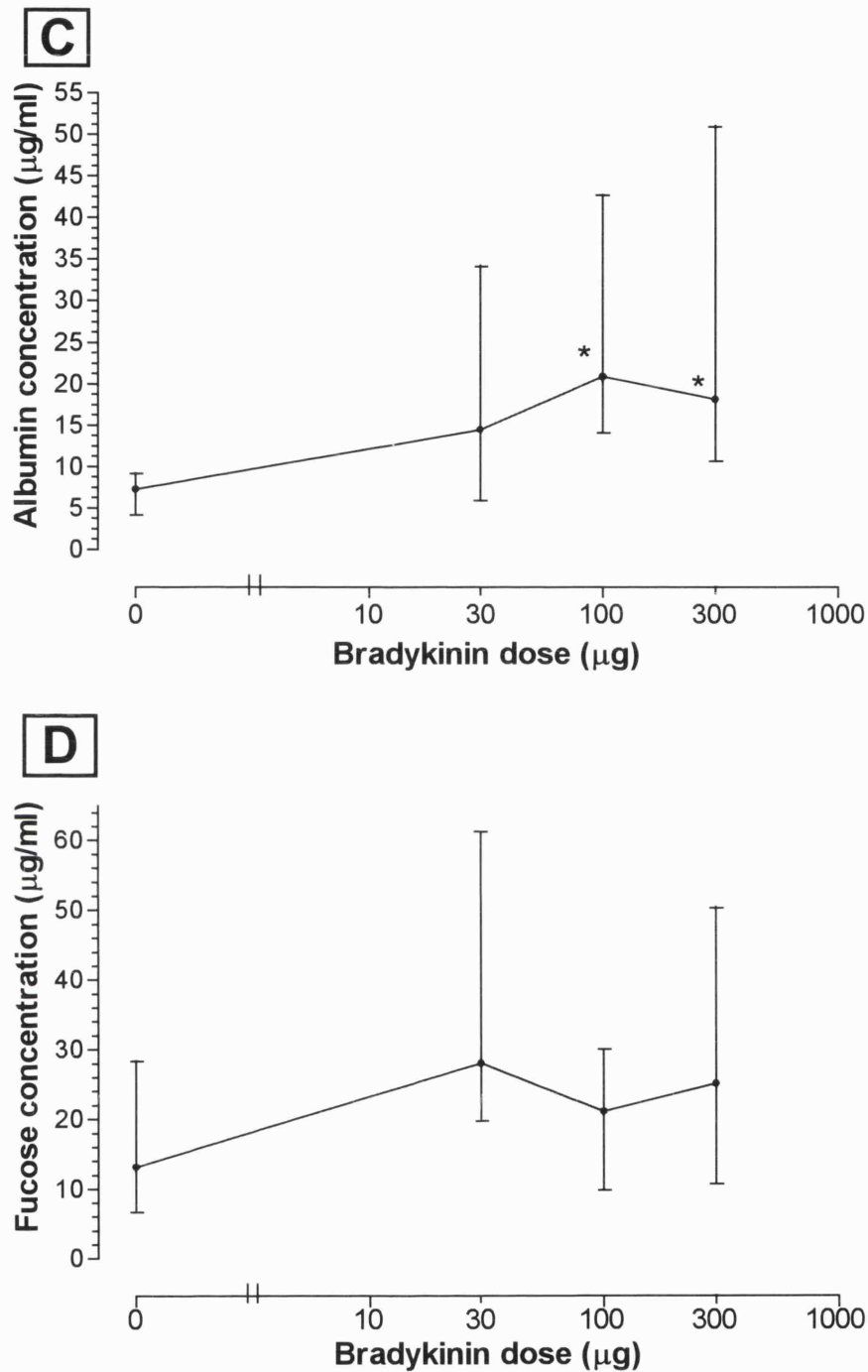


Figure 3.8 (C) and (D): Dose-response of albumin (C) and fucose (D) concentration in nasal lavage following increasing dose of bradykinin. The data are the medians of 8 subjects, with the 25th and 75th percentile values. The dose of bradykinin was plotted on a \log_{10} scale against the concentration of proteins in nasal lavage. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05). The concentration of the albumin increased following bradykinin challenges (Friedman's test, p-value < 0.05).

3.4 Discussion

Nasal provocation with methacholine induced a nasal sensation of warmth and a throbbing sensation; these responses are probably due to vasodilatation. Methacholine induced an increase in nasal mucosal secretory activity within 2 minutes and lasted for 18 minutes after challenge. The protein constituents of the nasal secretion induced by methacholine are a mixture of plasma proteins and glandular proteins as albumin (plasma origin), lysozyme and lactoferrin (serous cell origin) and mucous glycoprotein (mucous cells and goblet cells) are all increased following methacholine challenges. These observations are consistent with published observations that methacholine provocation induces nasal secretion (Sjogren *et al.*, 1988; Gawin *et al.*, 1991) and glandular protein secretion (Gawin *et al.*, 1991; Mullol *et al.*, 1992b; Naclerio & Baroody, 1995). Cholinergic fibres are found close to blood vessels, but are particularly numerous around the submucosal glands (Vecerina *et al.*, 1983; Katahashi *et al.*, 1997). Glandular secretion induced by methacholine can be blocked by treatment with non-selective muscarinic antagonists, atropine (Druce *et al.*, 1985) and ipratropium bromide (Baroody *et al.*, 1992; Borum *et al.*, 1996; Meltzer *et al.*, 1992; Naclerio *et al.*, 1995). *In vitro*, methacholine-induced MGP release was significantly inhibited by atropine, pirenzepine (M_1 receptor antagonist) and 4-diphenylacetoxy-N-methylpiperidine methiodine (4-DAMP, M_3 receptor antagonist) but not by M_2 receptor antagonists, gallamine and AF-DX116. This suggests that methacholine exerts its action upon activation of muscarinic receptors, M_1 and M_3 . Autoradiographic ligand binding studies indicate that M_1 and M_3 binding sites are present on submucosal glands, epithelium and endothelium in the nose (Mak & Barnes, 1990; Baraniuk *et al.*, 1992a; Okayama *et al.*, 1993). M_3 binding sites are also present on vessels (Baraniuk *et al.*, 1992a; Okayama *et al.*, 1993). Activation of M_3 receptors on the vessel induces vasodilatation which, in turn, increases blood flow. The increase in blood flow allows plasma protein to leak into the mucosa via fenestrated capillaries. This explains the presence of albumin in nasal lavage following methacholine provocation. The distribution of muscarinic receptors and cholinergic nerves appear to correlate with the effects of methacholine in nasal mucosa.

Methacholine is often used for nasal challenge due to its effect as a secretagogue, especially upon submucosal glands. It is, therefore, used in nasal challenges as an indicator of secretory response (Mygind & Dahl, 1996; Baroody *et al.*, 1996). In this study, a new technique used for quantification of the secretory responses by cotton buds, proved to be applicable and capable of detecting both, very small (baseline) secretion, and larger amounts of secretion induced by methacholine. Furthermore, serial determination of nasal secretions can be carried out with ease and rapidity so that the duration of the effect of methacholine and other challenge solutions can be measured.

Marked nasal responses were observed following histamine provocation, namely sneezing, itching and rhinorrhea. Histamine induced an increase in nasal secretion, which lasted for 14 minutes. Histamine challenge caused an increase in both plasma protein and glandular protein in nasal lavage fluid. Histamine is known to induce vasodilatation and vascular permeability (Ash & Schild, 1966), which allows plasma proteins to leak into the nasal lumen. Thus histamine produces a greater increase in albumin concentration than that caused by methacholine, because methacholine is only a vasodilator. The observation that histamine provocation induces albumin and glandular secretion is in agreement with findings by Mullol *et al.* (1992b) and Raphael *et al.* (1989b), that *in vivo*, histamine induced vascular permeability and glandular secretion. However, *in vitro*, histamine has been shown to have no effect on glandular secretion (Mullol *et al.*, 1992b). Autoradiographic studies have shown H₁ receptor binding sites exclusively in the endothelium of vessels (Okayama *et al.*, 1992). Therefore, histamine exerts its vascular effect by the activation of these H₁ receptors. In contrast, no specific binding could be observed in the submucosal glands or epithelium (Okayama *et al.*, 1992). This finding is not consistent with the *in vivo* increase in glandular proteins, lysozyme, lactoferrin and MGP, following histamine provocation in this study and in others, but is consistent with the lack of histamine effect on glandular secretion *in vitro*. It has been proposed that the glandular effect of

histamine *in vivo* is an indirect action of histamine (Mullol *et al.*, 1992b) and may be the result of sensory-parasympathetic reflexes (Raphael *et al.*, 1989b).

The main purpose of the experiments described in this chapter was to compare the effect of bradykinin with methacholine (a known nasal secretagogue) and histamine (a known inflammatory mediator). Nasal challenge with bradykinin induced nasal secretion, which lasted for 10 minutes. Unlike histamine, sneezing and itching were not observed with bradykinin. Bradykinin induced an increase in albumin but did not increase the release of the glandular proteins, lysozyme, lactoferrin and MGP. The increase in albumin caused by bradykinin was of greater magnitude than that produced by methacholine. Bradykinin is known to induce an increase in vascular permeability (Regoli & Barabe, 1980). Increase in vascular permeability allows plasma proteins to leak into the nasal lumen. Thus, bradykinin produces a greater increase in albumin concentration than that caused by methacholine, because methacholine is only a vasodilator. However, bradykinin, unlike methacholine and histamine, does not appear to stimulate serous and mucous cells. Bradykinin has been shown to induce an increase in nasal airway resistance (Austin & Foreman, 1994a; Rajakulasingan *et al.*, 1991 and 1993; Holmberg *et al.*, 1990). It is thought to do this partly by increasing the thickness of the mucosa through swelling and engorgement. Bradykinin is a potent vasodilator and acts upon endothelial cells to produce contraction which creates spaces within the endothelial wall. This allows plasma proteins to leak from blood vessels, thus increasing vascular permeability: leakage of plasma proteins into the surrounding tissues leads to oedema. Oedema together with vasodilatation contributes to thickening of the nasal mucosa and thus nasal obstruction. The vascular effects of bradykinin are presumably mediated through an activation of the kinin B₂ receptor subtype as the B₁ agonist, [Des-Arg⁹]-BK, was totally ineffective in inducing an increase in nasal airway resistance and vascular permeability (Austin & Foreman, 1994a; Rajakulasingam *et al.*, 1991; Churchill *et al.*, 1991). Bradykinin is a highly potent B₂ receptor agonist, and autoradiographic studies have revealed [¹²⁵I]-BK binding sites on the vascular smooth muscle and endothelium of all vessels in the human nasal mucosa, but they are absent on glands and epithelium (Baraniuk *et al.*, 1990e). These observations correspond to

the lack of glandular secretion following bradykinin provocation. Therefore bradykinin stimulates nasal secretion via its direct action on B₂ receptors and nasal blood vessels to induce vasodilatation and increase vascular permeability.

In conclusion, this chapter shows the difference in the effect of methacholine, histamine and bradykinin on the normal human nasal mucosa. The effects of methacholine are mainly on the secretion of serous and mucous glands, with little effect on nasal blood vessels. Histamine produced both vascular and glandular effects. The vascular effect of histamine was of greater magnitude than that of methacholine, as indicated by the amount of albumin in the lavages: this indicates that histamine induced both vasodilatation and vascular permeability, presumably via H₁ receptor activation. Bradykinin produced only an albumin response which, like histamine, was of greater magnitude than the response induced by methacholine. Bradykinin also induced vasodilatation and vascular permeability, presumably via activation of B₂ receptors located on the vessels. The lack of glandular response and central reflexes, sneeze and itching, in bradykinin provocation suggests that bradykinin does not activate neuronal reflexes in the nose. The effect of bradykinin is also consistent with it being a mediator of perennial rhinitis (Austin *et al.*, 1994c) since clinically in this disorder there is little nasal secretion, mainly blockage.

CHAPTER 4

THE EFFECT OF CETIRIZINE AND HOE 140 ON HISTAMINE-AND BRADYKININ-INDUCED NASAL SECRETION AND PROTEIN CONTENT IN NASAL LAVAGE.

4.1 Introduction

Antihistamines are the pharmacological cornerstones of the treatment of seasonal allergic rhinitis. Second-generation histamine H₁-receptor antagonists (cetirizine, terfenadine, loratidine and astemizole) are largely used in the treatment of allergic rhinitis due to the lower incidence of sedative effects compared to the first-generation antihistamines such as chlorpheniramine. Cetirizine was shown to be more effective than terfenadine and loratidine in suppressing histamine-induced wheal and flare reaction in the skin (Monroe *et al.*, 1997). Cetirizine produced a greater improvement in symptoms of seasonal allergic rhinitis than terfenadine (Lockey *et al.*, 1996). Cetirizine, 10 mg, once a day has been shown to improve symptom scores of patients with perennial allergic rhinitis (Murriss-Espin *et al.*, 1998) and seasonal allergic rhinitis (Lockey *et al.*, 1996), and after histamine challenge (Mansmann *et al.*, 1992). Pretreatment with cetirizine reduced an increase in nasal airway resistance induced by histamine (Hilberg *et al.*, 1995; Frossard *et al.*, 1997).

Bradykinin stimulates nasal responses via activation of B₂ receptor subtypes, as B₁ receptor agonists are inactive, both in normal and atopic subjects (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a). Furthermore, in a study involving binding displacement of [¹²⁵I]-Hoe 140, [des-Arg⁹]-BK (B₁ receptor agonist) and [des-Arg⁹]-Hoe 140 (B₁ receptor antagonist) failed to displace [¹²⁵I]-Hoe 140 binding while bradykinin, a potent B₂ agonist and B₂ receptor antagonists, such as [D-Arg⁰, Hyp³, D-Phe⁷]-BK (NPC 567) and Hoe 140 were able to displace [¹²⁵I]-Hoe 140 binding (Dear *et al.*, 1996b). Binding displacement together with autoradiographic studies of [¹²⁵I]-bradykinin (Baranuik *et al.*, 1990e) emphasise the role of B₂ receptors in bradykinin-

induced nasal responses. Hoe 140 is a very potent B₂ receptor antagonist. The effectiveness of Hoe 140 at inhibiting the action of bradykinin over other B₂ antagonists has been demonstrated in many tissue preparations *in vitro* (Hock *et al.*, 1991; Marceau *et al.*, 1994). In human nasal mucosa, NPC 567 was incapable of blocking the nasal response to bradykinin provocation (Pongracic *et al.*, 1991). Hoe 140 on the other hand was able to block the response to bradykinin challenges in normal volunteers (Austin *et al.*, 1994c; Proud *et al.*, 1995) and in antigen-induced nasal blockage (Austin *et al.*, 1994c).

The purpose of this study was to determine the effect of cetirizine and Hoe 140 on nasal secretion and plasma and glandular protein concentrations following histamine and bradykinin challenge.

4.2 Experimental protocol

For the following experiments, the study of nasal secretory activity was conducted on separate occasions from the study of the proteins released. Normal, healthy volunteers aged 20 to 40 years were used. No subject was taking any medication at the time of the experiment and in the week prior to the study. A double-blind, cross-over design was used in all of the protocols.

4.2.1 Effect of cetirizine on histamine-induced secretory response.

Each subject received pretreatment with placebo (lactose tablets) or cetirizine (10 mg tablets, a standard clinical dose) 3-4 hours prior to the experiment. The baseline nasal secretion was obtained prior to nasal challenge. Three minutes after the baseline measurement, histamine was then delivered into the nasal cavity by a handheld pump spray. The first measurement of nasal secretion following histamine challenge was taken 1 minute after the challenge. With 1 minute allowed for absorption of secretion into the cotton bud, further measurements were taken every 3 minutes. The sequence of the protocol is illustrated in Figure 4.1

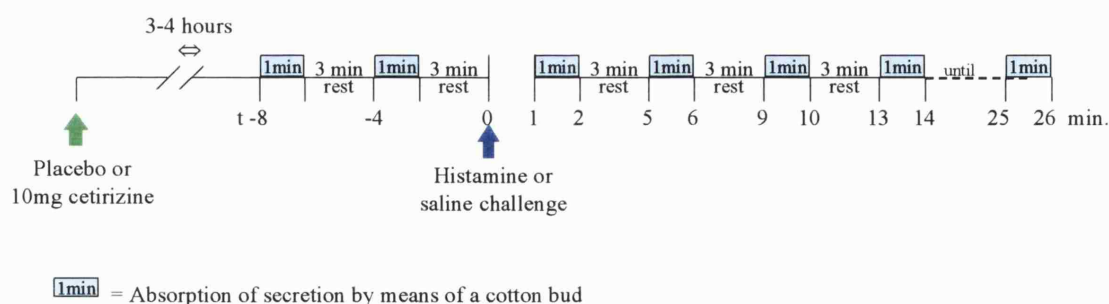


Figure 4.1: Protocol for investigating the effect of 10 mg cetirizine on histamine-induced nasal secretion.

Saline and 3 doses of histamine (100, 300 and 1000 µg) were used for the studies involving both placebo and cetirizine treatments. All subjects received both treatments on separate occasions, at least 24 hours apart.

4.2.2 Effect of cetirizine on histamine-induced lysozyme, lactoferrin, albumin and mucous glycoprotein secretion.

In the study of the protein release, each subject received pretreatment with placebo (lactose tablets) or cetirizine (10 mg tablets, a standard clinical dose) 3-4 hours prior to the experiment. At the beginning of the experiment nasal lavage was performed three times per nostril, prior to nasal challenge. The third lavage was used for the measurement of pre-challenge protein content in the lavage. Then histamine was delivered into the nasal cavity as in the secretory study. A nasal lavage was performed 10 minutes after the challenge. The concentration of lactoferrin, lysozyme, albumin and mucous glycoprotein (measured as fucose) in the lavage was determined by the methods which have already been described in chapter 2.

Saline and 3 doses of histamine (100, 300 and 1000 µg) were used for the studies involving both placebo and cetirizine treatment. All subjects received both treatments on separate occasions, at least 24 hours apart.

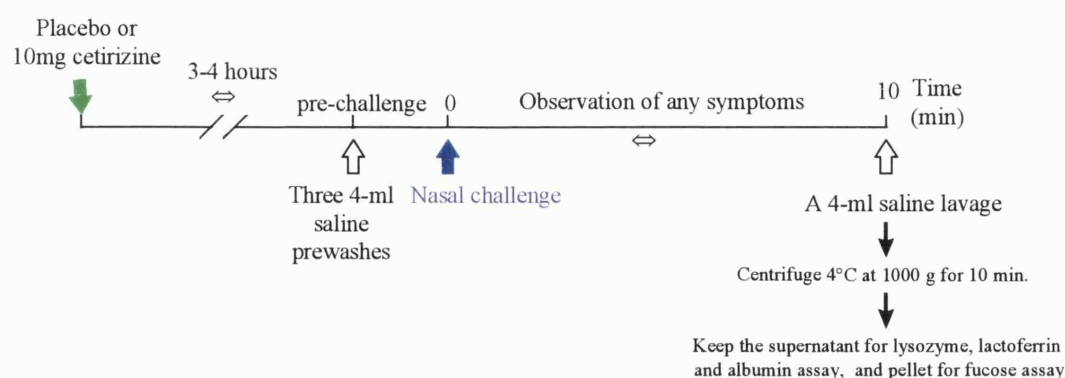


Figure 4.2: Protocol for investigating the effect of cetirizine on histamine-induced secretion of lactoferrin, lysozyme, albumin and mucous glycoprotein.

4.2.3 Effect of Hoe 140 on bradykinin-induced secretory response.

In this study, each subject received pretreatment with saline solution, as placebo, or Hoe 140, delivered into the nasal cavity by a handheld pump spray, 3 minutes prior to nasal challenge. Hoe 140 at a dose of 200 μg was used since this dose has been shown to inhibit the effect of bradykinin on nasal patency (Austin *et al.*, 1994c).

The measurement of baseline nasal secretion was first taken, and then followed by a treatment with placebo or Hoe 140. Three minutes after the treatment, bradykinin was then delivered into the nasal cavity. The first measurement of nasal secretion following bradykinin challenge was taken 1 minute after the challenge. With 1 minute allowed for absorption of secretion into the cotton bud, further measurements were taken every 3 minutes. The sequence of the protocol is illustrated in Figure 4.3

Bradykinin 30, 100 and 300 μg were used in the study. Again, each subject received both saline and Hoe 140 treatment, and all of the bradykinin doses and saline in both protocols.

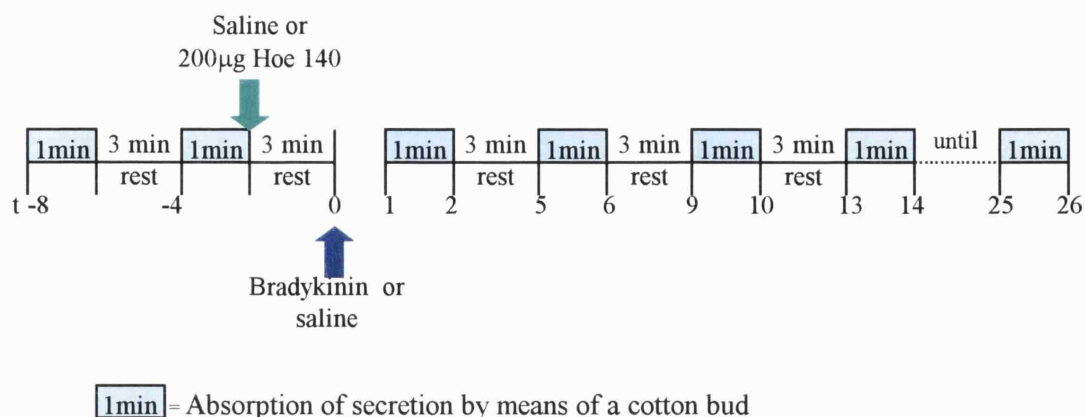


Figure 4.3: Protocol for investigating the effect of 200 µg Hoe 140 on bradykinin-induced nasal secretion.

4.2.4 Effect of Hoe 140 on bradykinin-induced lysozyme, lactoferrin, albumin and mucous glycoprotein secretion.

In the study of protein secretion, at the beginning of each experiment nasal lavage was performed three times per nostril, prior to any nasal treatment with saline solution or Hoe 140. The third lavage was used for the measurement of pre-challenge protein content in the lavage. After the third lavage was collected, saline or Hoe 140 was delivered into the nasal cavity. The nasal cavity was then challenged with saline or bradykinin 3 minutes after saline or Hoe 140 pretreatment. A nasal lavage was performed 10 minutes after the challenge with saline or bradykinin. The concentration of lactoferrin, lysozyme, albumin and mucous glycoprotein (measured as fucose) in the lavage was determined by the methods that have already been described in chapter 2. The sequence of the protocol is illustrated in Figure 4.4. As with the secretory response, 30, 100 and 300 µg of bradykinin were used in the study. Each subject received both saline and Hoe 140 treatment, and all of the bradykinin doses and saline in both protocols.

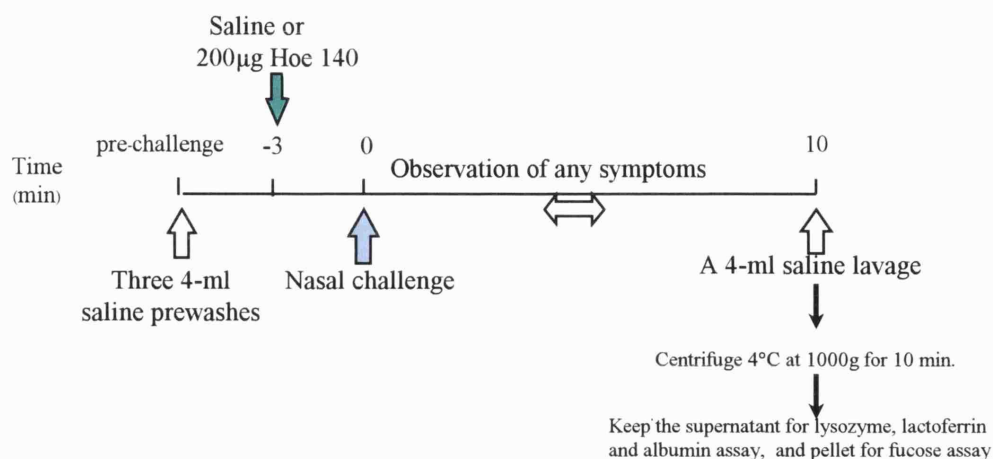


Figure 4.4: Protocol for investigating the effect of Hoe 140 on bradykinin-induced secretion of lactoferrin, lysozyme, albumin and mucous glycoprotein.

4.2.5 Record of symptoms

The number of sneezes was counted and recorded after every nasal challenge. Any other symptoms, such as itching, pain and sore throat, experienced were given voluntarily by the subjects and noted.

4.2.6 Data analysis

The medians and percentiles were determined for all parameters; the rate of nasal secretion (mg/min), the area under the curve (AUC) of secretion response over 26 minutes observation and protein concentration in the nasal lavage. A Friedman's test was performed to compare the effects of a series of increasing doses of challenge solutions on a group of individuals. Dunn's test was performed following the Friedman's test when there was a statistically significant difference in the treatments, in order to determine exactly which treatment has an effect in comparison to saline. This test was performed on the secretory response at each time point, on the AUC and on protein content in the nasal lavage. A $p\text{-value} \leq 0.05$ was considered to be significant.

To assess differences between the two treatment groups (placebo- and cetirizine-treated, and placebo- and Hoe 140-treated), Wilcoxon signed rank test with Bonferroni's correction factor was employed. A p-value ≤ 0.017 was considered to be significant.

4.3 Results

4.3.1 *Effect of cetirizine on histamine-induced secretory response.*

Eight subjects took part in the study. Each subject received both placebo and cetirizine treatments, and three doses of histamine or saline for each treatment. The time courses of histamine with placebo treatment and with cetirizine treatment are shown in figure 4.5. In the placebo-treated group, the secretory response to histamine reached maximal intensity within 2 minutes and decreased to baseline levels after 10 minutes (Friedman's test, p-values < 0.05 at $t = 2, 6$ and 10 minutes), figure 4.5 (A). The secretory response induced by 1000 μg histamine was significantly greater than that of saline at $t = 2$ minutes (Dunn's test, p-values < 0.05). At $t = 6$ minutes, 300 and 1000 μg histamine induced a significant increase in the response in comparison with saline (Dunn's test, p-values < 0.05). At $t = 10$ minutes, 1000 μg histamine still induced a significant increase in the response in comparison with saline (Dunn's test p-values < 0.05). In the cetirizine-treated group (figure 4.5 (B)), on the other hand, all doses of histamine failed to induce a significant increase in nasal secretion at each time point (Friedman's test, p-values > 0.05).

In addition to the difference in the secretory response, several differences between the two treatments in terms of the clinical symptoms induced by histamine were noted. The most notable difference was in the number of sneezes (Table 4.1). There were no sneezes recorded after subjects were treated with cetirizine. No other symptoms were observed in both treatment groups. There was no significant difference in the secretory

response to saline challenge with or without cetirizine treatment (Wilcoxon test with Bonferroni's correction factor applied, p-values > 0.017).

Table 4.1: The median number of sneezes following histamine challenge in 8 subjects.

Histamine dose	Placebo treatment			Cetirizine treatment
	Median number of sneezes	25% percentile	75% percentile	Median number of sneezes
100 µg	1	0	2	0
300 µg	1	0	4	0
1000 µg	2	1.5	7.5	0

The changes in AUC for the 26-minute observation period following histamine challenge with placebo (black line) and cetirizine (green line) pretreatment are presented in figure 4.6. In the placebo group, histamine induced an increase in the AUC (Friedman's test, p-value < 0.02) with 1000 µg histamine inducing a significant increase in the response compared with that of saline (Dunn's test, p-value < 0.05). Histamine failed to induce any significant increase in AUC when it was applied after cetirizine pretreatment (Friedman's test, p-value > 0.1). When the AUC of each histamine concentration was compared between the treatment groups, pretreatment with cetirizine significantly reduced the increase in AUC induced by 1000 µg histamine as indicated by Wilcoxon test with Bonferroni's correction factor applied (p-value < 0.008). Although pretreatment with cetirizine also reduced the increase in AUC induced by 100 and 300 µg histamine, the changes that occurred with the treatment were not greater than would be expected by chance (Wilcoxon test, p-values > 0.017 for both doses).

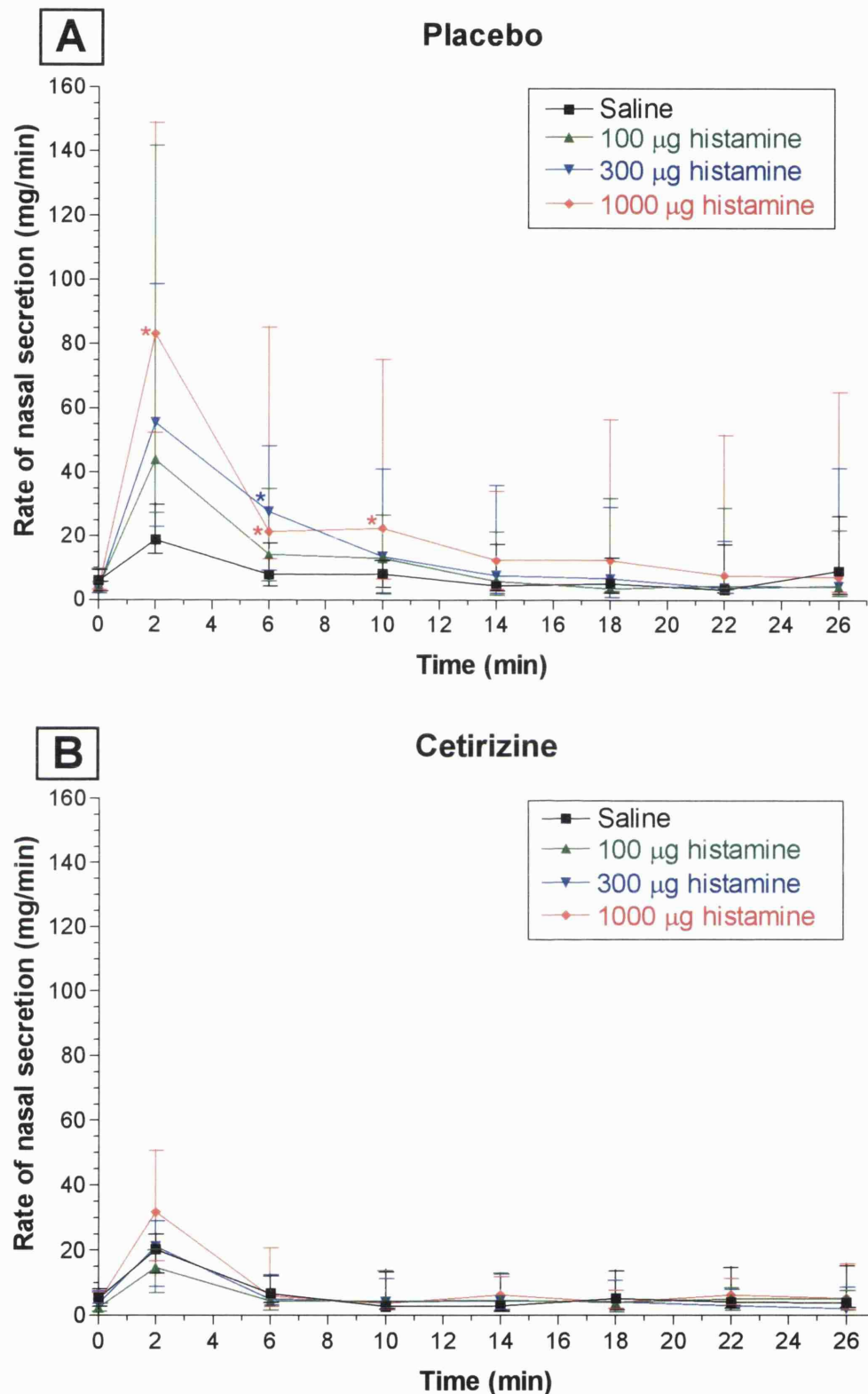


Figure 4.5 (A) and (B): The effect of histamine, over 26 minutes, on the secretory response of eight subjects. Subjects were treated with placebo (A) or 10 mg cetirizine (B) prior to nasal challenges. The data are the median values with the 25th and 75th percentile values. A statistically significant difference from the saline value at each time point is indicated by * (* p-value < 0.05).

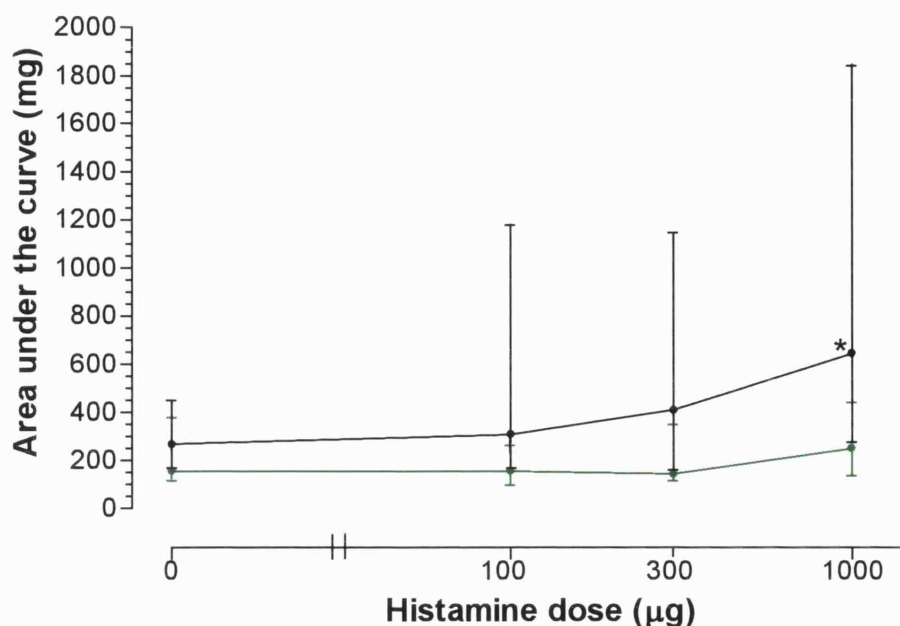


Figure 4.6: Dose-response relationship of nasal secretion to increasing dose of histamine following placebo treatment (black line) and cetirizine treatment (green line) are presented. The data are the medians of 8 subjects, with the 25th and 75th percentile values. The dose of histamine was plotted on a log₁₀ scale against the secretory response over 26 minutes. A statistically significant difference from the saline value for each treatment group is indicated by * for the placebo-treated or * for the cetirizine-treated group (*or* p-value < 0.05).

4.3.2 Effect of cetirizine on histamine-induced lysozyme, lactoferrin, albumin and mucous glycoprotein secretion.

Seven subjects took part in this study. In the two treatment groups, saline provocation produced no clinical symptoms, however, there was a difference in the number of sneezes following the administration of histamine. Following pretreatment with placebo, histamine provocation induced sneezing in all subjects with the highest number at the highest dose. Sneezing was not observed after histamine provocation with cetirizine pretreatment. Table 4.2 shows the median number of sneezes following histamine challenge in both treatment groups. No other symptoms were observed following histamine challenge in both treatment groups. The protein results from the placebo- and cetirizine-treated groups are presented as dose-response curves with the median value of protein content in the nasal lavage from 7 subjects.

Table 4.2: The median number of sneezes following histamine challenge in 7 subjects.

Histamine dose	Placebo treatment			Cetirizine treatment
	Median number of sneezes	25% percentile	75% percentile	Median number of sneezes
100 µg	1	0	2	0
300 µg	2	0	3	0
1000 µg	3	2	6	0

There was an increase in lactoferrin concentration following histamine challenges in both treatment groups, (Friedman's test, p-values < 0.03 for placebo- and cetirizine-treated group), figure 4.7 (A). In both treated groups there was a significant increase in lactoferrin concentration after 1000 µg histamine (Dunn's test, p-values < 0.05). There was no significant difference between the two treatments (placebo and cetirizine), in histamine-induced lactoferrin secretion at each histamine dose (Wilcoxon test with Bonferroni's correction factor applied, p-values > 0.017).

There was an increase in lysozyme concentration following histamine challenges in the placebo-treated group (Friedman's test, p-value < 0.02), figure 4.7 (B), black line. Analysis by the Dunn's test with respect to the saline value indicated that histamine induced a significant increase in lysozyme secretion at 300 and 1000 µg doses (p-values < 0.05) following placebo pretreatment. Pretreatment with cetirizine inhibited the histamine-induced an increase in lysozyme content (Friedman's test, p-values > 0.1) figure 4.7 (B), green line. However, there was no significant difference between the two treatments (placebo and cetirizine), in the histamine-induced lysozyme secretion at each histamine concentration (Wilcoxon test with Bonferroni's correction factor applied, p-values > 0.017).

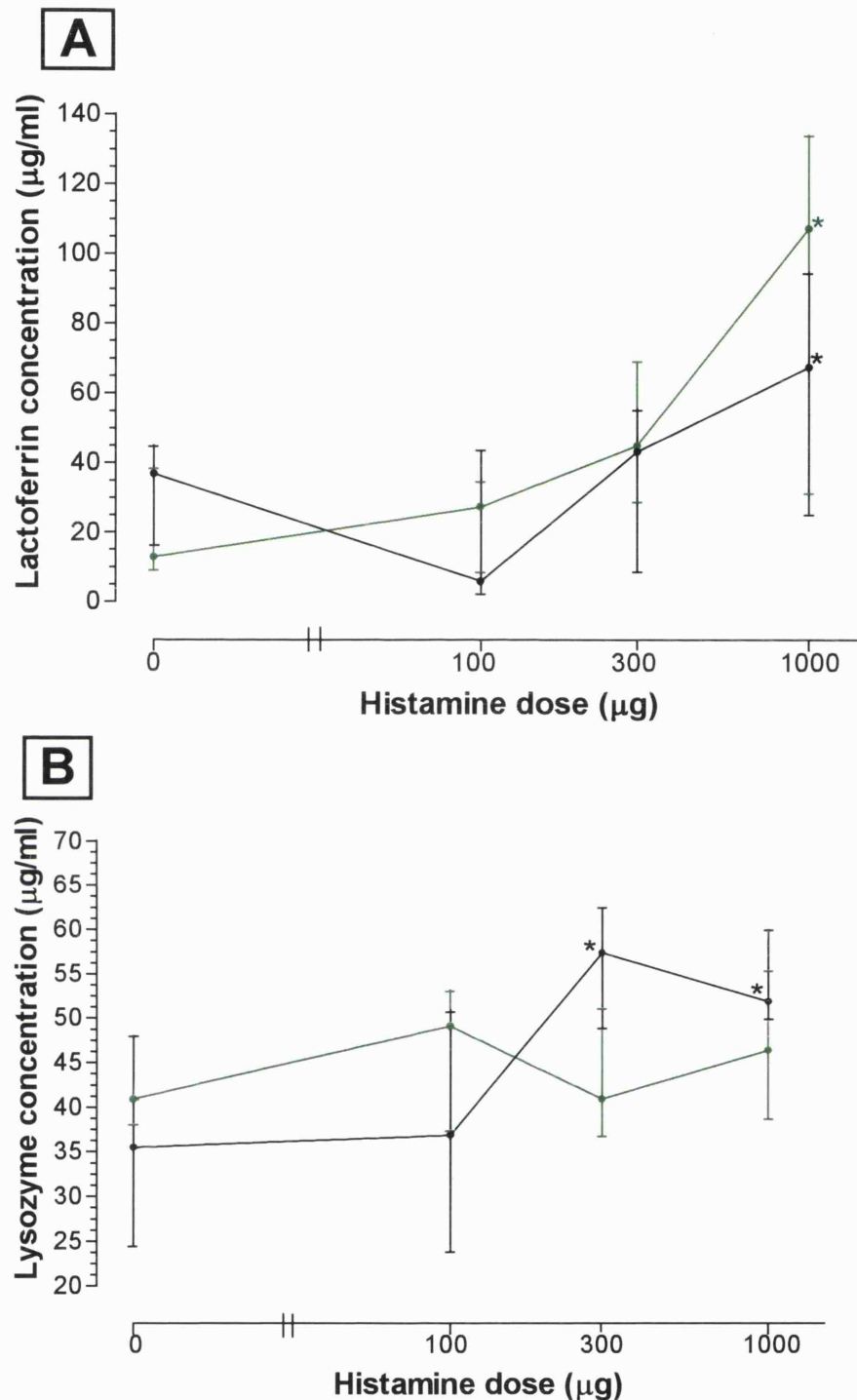


Figure 4.7 (A) and (B): Dose-response relationship of lactoferrin (A) and lysozyme (B) to an increasing dose of histamine following placebo treatment (black line) and cetirizine treatment (green line). The data are expressed as the medians of 7 subjects, with the 25th and 75th percentile values. A statistically significant difference from the saline value for each treatment group is indicated by * for the placebo-treated or * for the cetirizine-treated group (*or* p-value < 0.05). Histamine induced an increase in lactoferrin release in both treatment groups and in lysozyme release in the placebo treatment group. No statistically significant difference in the response to the same histamine dose was seen between the two treatments.

Figure 4.8 (A) and (B) show the effect of histamine on albumin and fucose content in the nasal lavage. In the placebo-treated group, histamine induced an increase in albumin secretion (Friedman's test p -value < 0.02). The increase in albumin secretion was significant at 300 and 1000 μg histamine (Dunn's test, p -values < 0.05), figure 4.8 (A) black line. Following cetirizine pretreatment, histamine failed to produce an increase in the content of albumin, figure 4.8 (A) green line, (Friedman's test, p -values > 0.1). There was a significant decrease in histamine-induced albumin secretion after cetirizine pretreatment compared with placebo pre-treatment, figure 4.9 (A). The effect of cetirizine on histamine-induced albumin secretion was significant at a histamine dose of 300 μg (Wilcoxon test, p -value ≤ 0.006). Although there was a decrease in histamine-induced albumin secretion after cetirizine pretreatment at a histamine dose of 1000 μg (Wilcoxon test, p -value ≤ 0.03), with Bonferroni's correction factor applied, the change that occurred with the treatment was not greater than would be expected by chance.

In the placebo-treated group, figure 4.8 (B) black line, there was an increase in fucose concentration (Friedman's test, p -value < 0.01). The increase in fucose was significant at 300 and 1000 μg histamine in comparison to saline (Dunn's test, p -values < 0.05). Cetirizine inhibited the histamine-induced increase in fucose concentration; figure 4.8 (B) green line, (Friedman's test, p -value > 0.1). However, when the fucose content in nasal lavage induced by the same dose of histamine, but after different treatments, was compared, there was no significant difference in fucose content between the two treatments (Wilcoxon test with Bonferroni's correction factor applied, p -values > 0.017).

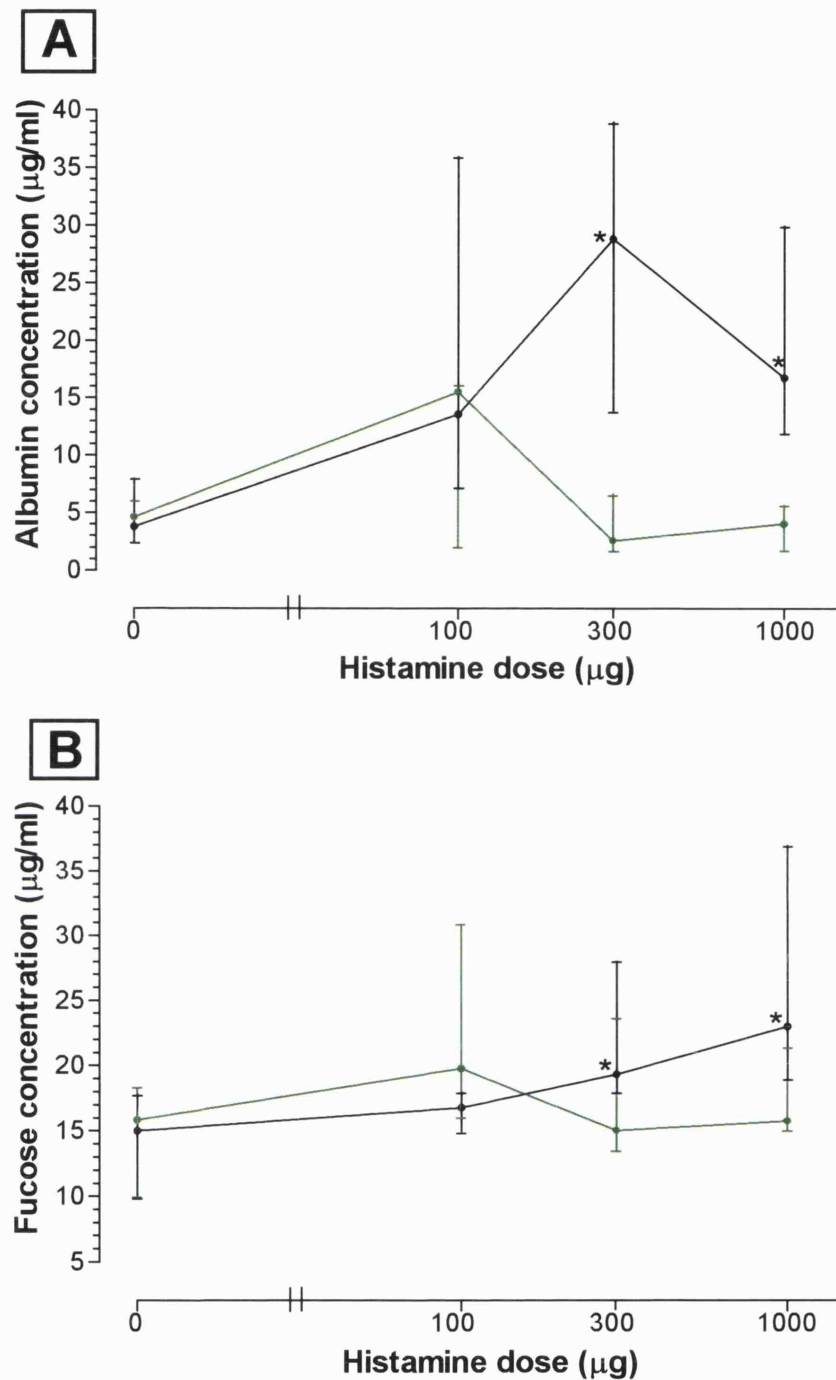


Figure 4.8 (A) and (B): Dose-response relationship of albumin (A) and fucose (B) to an increasing dose of histamine following placebo treatment (black line) and cetirizine treatment (green line). The data are expressed as the medians of 7 subjects, with the 25th and 75th percentile values. A statistically significant difference from the saline value for each treatment group is indicated by * for the placebo-treated or * for the cetirizine-treated group (*or* p -value < 0.05). Histamine induced an increase in albumin and fucose content after placebo treatment but not cetirizine treatment.

4.3.3 Effect of Hoe 140 on bradykinin-induced secretory response.

Figure 4.9 displays the secretory response at each time point following bradykinin challenge, with saline (A) and 200 µg Hoe 140 (B) pretreatment. The study was carried out on nine subjects who received intranasal challenges with saline or 30, 100 and 300 µg bradykinin; each challenge following 3 min after pretreatment with either saline or 200 µg Hoe 140. Six of nine subjects complained of nasal discomfort, such as sore throat and nasal pain, following 100 and 300 µg bradykinin in the saline pretreatment group. No other symptoms were observed. In the saline pretreatment group, bradykinin induced an increase in nasal secretion at 2, 6 and 10 minutes after the challenge (Friedman's test, p -value < 0.03 at $t=2$ minutes; p -values < 0.03 at $t=6$ and 10 minutes), figure 4.9 (A). Only 300 µg bradykinin produced a significant increase in the nasal secretion during 2 to 10 minutes (Dunn's test, p -values < 0.05). Nasal challenges with the same concentration of bradykinin after pretreatment with Hoe 140, however, failed at each time point to produce a significant increase in the response from that of saline (Friedman's test, p -values > 0.1), figure 4.9 (B). No other symptoms were observed.

Following saline pretreatment, bradykinin induced an increase in AUC of secretory response over a 26-minute period of observation (Friedman's test, p -value ≤ 0.001), with 300 µg of bradykinin inducing a significant increase in the response from that of saline (Dunn's test, p -value < 0.05). This increase in AUC after the same dose of bradykinin was inhibited after pretreatment with Hoe 140 (Friedman's test, p -value > 0.1), figure 4.10. However, there was no significant difference in the responses between the two treatments (Wilcoxon test with Bonferroni's correction factor applied, p -values > 0.017).

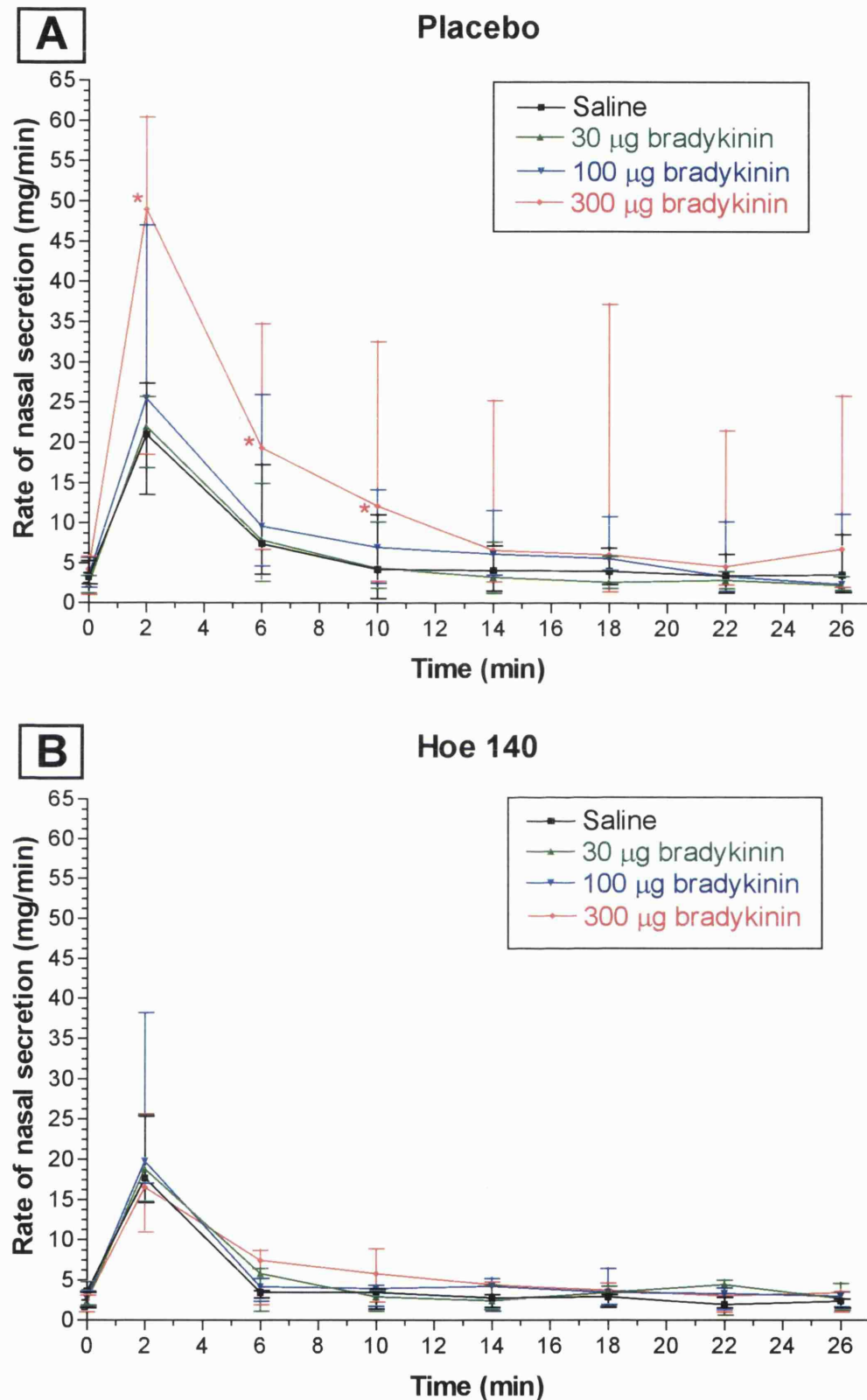


Figure 4.9 (A) and (B): The duration of effect of bradykinin over 26 minutes on the secretory response of nine subjects. Subjects were treated with placebo (A) or 200 μ g Hoe 140 (B). The data are the median values, with the 25th and 75th percentile values. A statistically significant difference from the saline value at each time point is indicated by * (* p-value < 0.05).

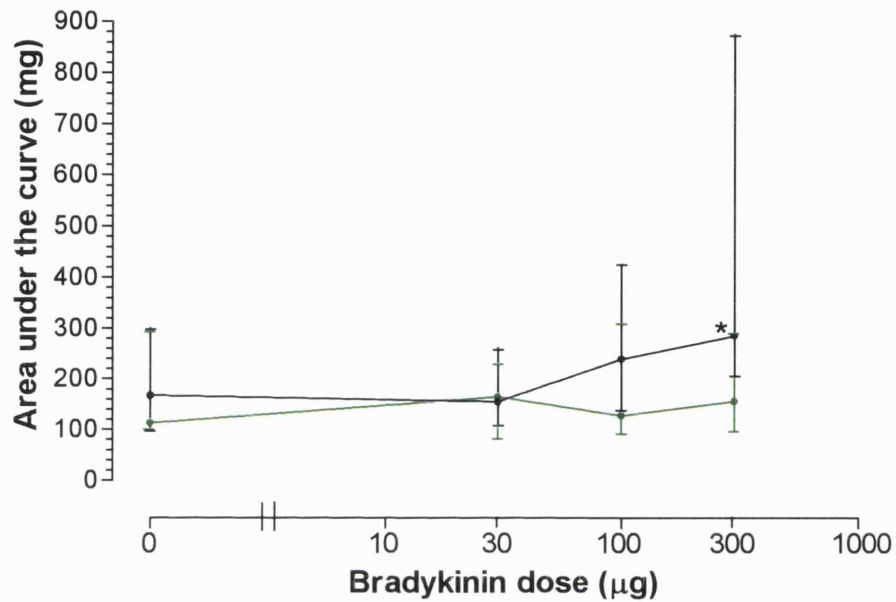


Figure 4.10: Dose-response relationship of nasal secretion to increasing dose of bradykinin following placebo treatment (black line) and 200 μg Hoe 140 treatment (green line) are presented here. The data are the medians with the 25th and 75th percentile values. The dose of bradykinin was plotted on a \log_{10} scale against the secretory response over 26 minutes. A statistically significant difference in the response to that of saline is indicated by * (* p-value < 0.05).

4.3.4 Effect of Hoe 140 on bradykinin-induced lysozyme, lactoferrin, albumin and mucous glycoprotein secretion.

Ten subjects took part in this study. Six out of ten subjects complained of nasal discomfort, such as sore throat and nasal pain following bradykinin provocation in the control group. No other symptoms were observed. No clinical symptoms were observed in the Hoe 140-treated group. The protein content in the nasal lavage of saline- and Hoe 140-treated groups following bradykinin provocation is presented as a dose-response relationship. Data are presented as the median of the protein concentration of 10 subjects. In figure 4.11 (A) and (B), in both control (black line) and Hoe 140 (green line) groups, bradykinin failed to produce any significant increase in lactoferrin and lysozyme secretion (Friedman's test, p -values > 0.5 for both lactoferrin and lysozyme).

Figure 4.12 (A) displays the effect of bradykinin challenge on albumin concentration in the nasal lavage, given 3 minutes after treatment with saline or Hoe 140. In both control (black line) and Hoe 140 (green line) treatment groups, bradykinin induced an increase in albumin content (Friedman's test, p -values ≤ 0.001), with 100 and 300 μg bradykinin inducing significantly greater albumin response than that of saline (Dunn's test, p -values < 0.05). When the responses to the same bradykinin doses were compared, pretreatment with Hoe 140, 3 minutes prior to bradykinin challenge, led to a significant reduction in the albumin concentration induced by 30 and 300 μg bradykinin (Wilcoxon test with Bonferroni's correction factor applied, p -values < 0.017). This reduction in the albumin content gave a displacement of the log dose-response curve of bradykinin-induced albumin secretion, figure 4.12 (A). Like lysozyme and lactoferrin responses, bradykinin failed to induce a significant increase in fucose after either treatment (Friedman's test, p -values > 0.3), figure 4.12 (B).

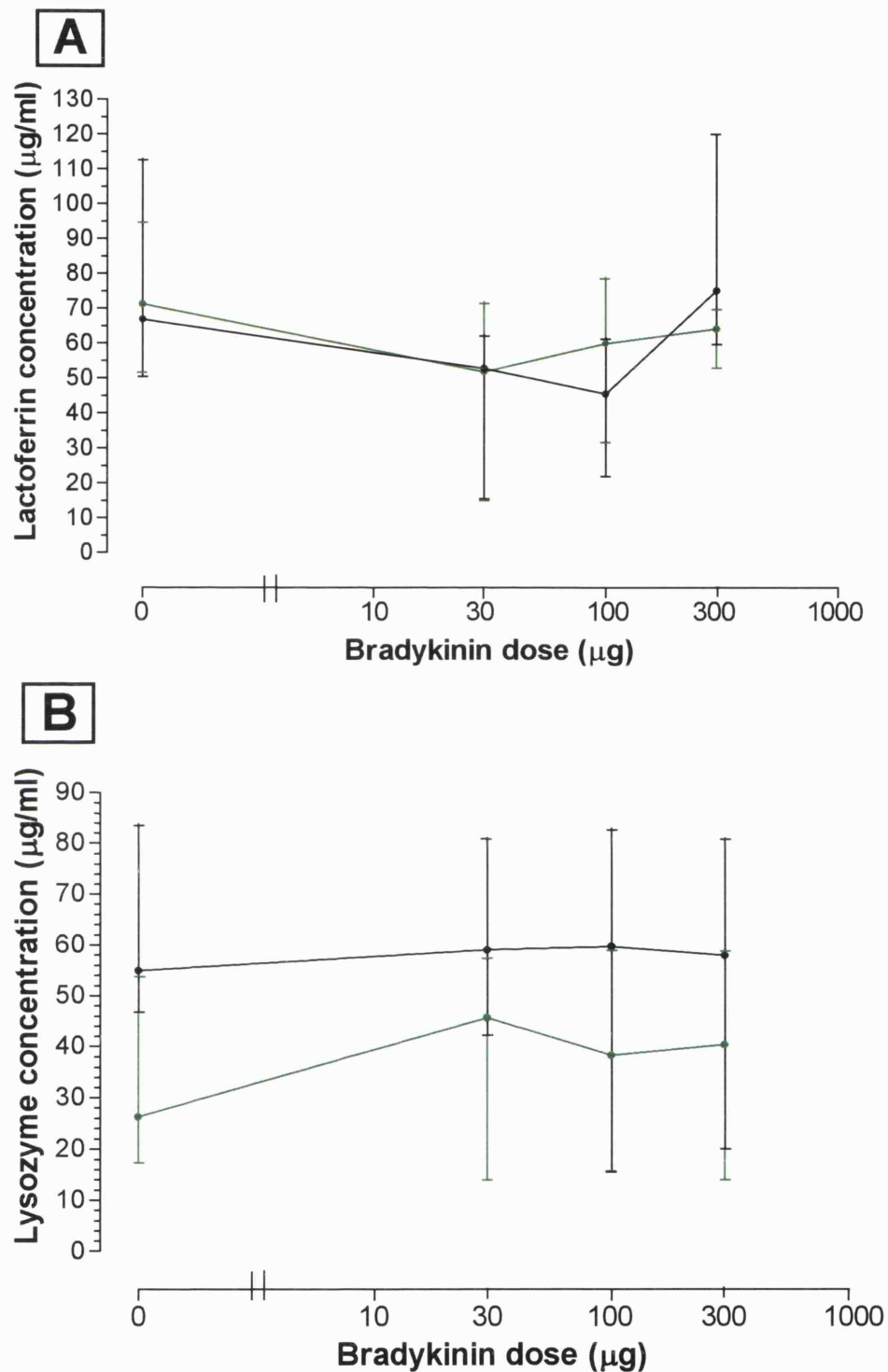


Figure 4.11(A) and (B): Dose-response relationship of lactoferrin (A) and lysozyme (B) to an increasing dose of bradykinin following saline treatment (black line) and Hoe 140 treatment (green line). The data are expressed as the medians of 10 subjects, with the 25th and 75th percentile values. The dose of bradykinin was plotted on a log₁₀ scale against the concentration of proteins in nasal lavage. A statistically significant difference from the saline value of each treatment group will be indicated by * (* p-value < 0.05). Bradykinin has no effect on lactoferrin and lysozyme concentration in nasal lavage.

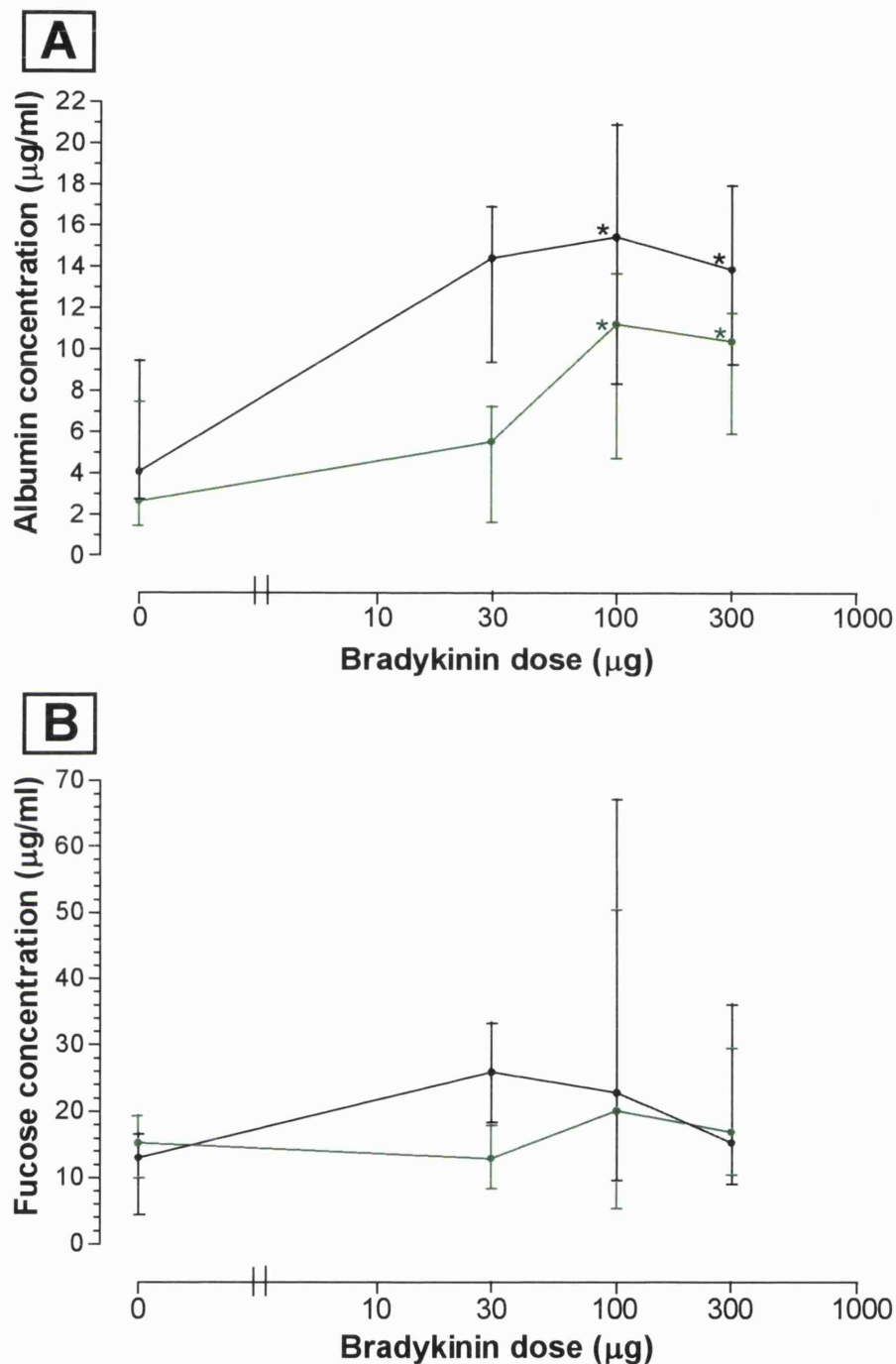


Figure 4.12 (A) and (B): Dose-response relationship of albumin (A) and fucose (B) to an increasing dose of bradykinin following placebo treatment (black line) and Hoe 140 treatment (green line). The data are expressed as the medians of 10 subjects, with the 25th and 75th percentile values. The dose of bradykinin was plotted on a log₁₀ scale against the concentration of albumin in nasal lavage. A statistically significant difference from the saline value of each treatment group is indicated by * for placebo-treated or * for the Hoe 140-treated group (*or* p-value < 0.05). Bradykinin induced an increase in albumin concentration in both treatment groups.

4.4 Discussion

In this study the effect of nasal challenge with histamine and bradykinin in the placebo treatment group is consistent with that reported previously in chapter 3. However the absolute values obtained in this study and the previous chapter are different. These differences may be due to factors such as subject variability, variability in the assay conditions, handling of samples before (storage conditions) and during the assay (dilution).

In this chapter, with the placebo treatment group, histamine induced an increase in nasal secretion. Pretreatment with 10 mg cetirizine blocked the histamine-induced increase in the nasal secretion response. Another noticeable nasal response that was inhibited by cetirizine pretreatment, was the number of sneezes. Cetirizine was able to block histamine-induced sneezing. Other studies have shown that cetirizine significantly reduced allergen-induced sneezing in experimentally-induced allergic reactions of rhinitis subjects (Naclerio *et al.*, 1989; Togias *et al.*, 1989), and improved symptom scores of rhinitis patients with perennial and seasonal allergic rhinitis (Murriss-Espin *et al.*, 1998; Ciprandi *et al.*, 1997a; Lockey *et al.*, 1996). Another less potent H₁ antagonist, terfenadine, is also able to reduce the number of sneezes following histamine provocation (Skoner *et al.*, 1991; Naclerio *et al.*, 1990). This indicates that the process leading to sneezing is a consequence of H₁ activation.

Another nasal response that is mediated through the H₁ receptor is an increase in vascular permeability. Pretreatment with cetirizine markedly reduced the level of albumin in nasal lavage following histamine provocation. Cetirizine also reduced the albumin level following nasal allergen response (Baroody *et al.*, 1989; Naclerio *et al.*, 1989). The reduction in albumin levels indicates that cetirizine blocks an increase in vascular permeability and suggests that histamine exerts its vascular effect via the activation of H₁ receptors located on nasal blood vessels. Cetirizine binds to H₁ receptors on the blood vessels and prevents the binding of histamine, thus inhibiting

vasodilatation and contraction of endothelial cells: the process that leads to increased vascular permeability. Histamine-induced vasodilatation is mediated through activation of H₁ receptors and cetirizine blocks the effect of histamine-induced increases in nasal airway resistance which is mainly caused by dilatation of blood vessels (Austin & Foreman, 1994b; Braunstein *et al.*, 1992; Wood-Baker *et al.*, 1996).

Histamine-induced an increase in lactoferrin, lysozyme and fucose concentration in the placebo-treated group and this is consistent with the study in chapter 3. The effect of cetirizine on glandular secretion is, on the other hand, not as clear-cut as that observed for plasma protein (albumin). Cetirizine had no effect on lactoferrin levels in the nasal lavage following histamine provocation. Histamine at 300 and 1000 µg significantly induced an increase in lysozyme and fucose levels, and cetirizine inhibited the increase in lysozyme and fucose levels. However, when the responses induced by the same dose of histamine were compared between the two treatments, there was no statistically significant difference in the responses between the treatments. Cetirizine, therefore, may only partially inhibit glandular secretion. Histamine has been shown to have a secretory effect through H₂ receptor activation such as in gastric-acid secretion (Black *et al.*, 1972). H₂ receptor mRNA has been shown to be localised in the nasal epithelium, serous cells and mucous cells (Hirata *et al.*, 1999).

Histamine-induced glandular secretion has been proposed to be an indirect action of histamine since no binding site has been identified on submucosal glands. It was suggested that the glandular secretion induced by histamine is mediated by neuronal reflexes, since histamine failed to induce glandular secretion from human nasal mucosal explants (Mullol *et al.*, 1992b), and histamine challenges on one side of the nose (ipsilateral side) induced contralateral secretion (secretion from the non-challenge side) as well as the ipsilateral secretion (Raphael *et al.*, 1989b; Naclerio *et al.*, 1992b). Whether or not H₁ receptor stimulation makes some contribution to neuronal reflex-induced glandular secretion cannot be concluded from this study. Cetirizine has been reported to have additional anti-inflammatory properties. These include a reduction in ICAM-1 (intracellular adhesion molecule) expression on nasal epithelial cells (Ciprandi

et al., 1997a and 1997b) and LTC₄ formation (Naclerio *et al.*, 1991b; Baroody *et al.*, 1989; Togias *et al.*, 1989). The reduction in ICAM-1 expression may in turn reduce an infiltration of inflammatory cells. These additional properties of cetirizine may contribute to its effectiveness in treatment of allergic rhinitis.

In this study the effect of nasal challenge with bradykinin is consistent with that reported previously (chapter 3). Bradykinin induced an increase in nasal secretion and albumin levels, but not in lysozyme, lactoferrin and fucose levels. Pretreatment with 200 µg Hoe 140 significantly inhibited bradykinin-induced nasal secretion. The same observation was made with the albumin response. Pretreatment with Hoe 140 significantly reduced albumin levels in nasal lavage. Hoe 140, therefore, caused a shift in the dose-response curve of a nasal secretion and albumin. Bradykinin-induced rises in vascular permeability, as indicated by albumin levels, are inhibited by Hoe 140, a potent B₂ antagonist, since both bradykinin and Hoe 140 are competing for the same B₂ receptors, located exclusively on vessels. Hoe 140 is effective at reducing the vascular effects of bradykinin and it reduces the increase in bradykinin-induced nasal airway resistance in normal subjects (Austin *et al.*, 1994c; Proud *et al.*, 1995).

Bradykinin has been suggested to be an important mediator of allergic rhinitis because there was a greater nasal response to bradykinin provocation in rhinitics than that seen in normal non-atopic subjects (Brunnee *et al.*, 1991; Baraniuk *et al.*, 1994a and 1994b; Riccio & Proud, 1996). Bradykinin has been shown to induce greater symptom scores in allergic rhinitic subjects than in normal subjects. Sneezing was also observed in a majority of rhinitic subjects following bradykinin provocation (Riccio & Proud, 1996). Bradykinin induces glandular secretion in chronic allergic rhinitis (Baraniuk *et al.*, 1994a). Bradykinin induces dose-dependent plasma leakage into the nasal cavity, which is significantly greater in allergic rhinitic subjects than in normal subjects (Brunnee *et al.*, 1991). The difference in the response to bradykinin in allergic rhinitis indicates an alteration in nasal responses to bradykinin. Whether the alteration in nasal response involves an increase in B₂ receptors in allergic rhinitis cannot be deduced at this stage due to a lack of experimental evidence. The B₁ receptor has been reported to

be upregulated in many chronic disease states. In allergic rhinitis, it was demonstrated that the B₁ agonist [Des-Arg⁹]-bradykinin has no effect on symptom scores and nasal airway resistance in rhinitis subjects (Rajakulasingam *et al.*, 1991). The study provides evidence that B₁ receptors are not upregulated in rhinitis. Hoe 140 was able to reduce nasal blockage induced by antigen challenge in perennial allergic rhinitis (Austin *et al.*, 1994c; Proud *et al.*, 1995). Hoe 140 may therefore be useful in the treatment of allergic rhinitis.

In conclusion, cetirizine inhibited the histamine-induced increase in albumin secretion and sneezing, with partial inhibitory effects on the amount of nasal secretion and glandular secretion. The reduction in the albumin release indicates that the histamine-induced vascular response is H₁ receptor mediated. The ability of cetirizine to inhibit sneezing and partially inhibit the glandular response indicated that a histamine-induced neuronal reflex may be mediated through an H₁ receptor. Bradykinin, on the other hand, has no effect on glandular responses and central reflexes (sneeze), but only on vascular responses. The bradykinin-induced vascular response was inhibited by the B₂ antagonist, Hoe 140, and this indicated that the vascular response is mediated via B₂ receptor activation.

CHAPTER 5

THE EFFECT OF UNILATERAL HISTAMINE CHALLENGES ON NASAL SECRETION AND PROTEIN CONTENT IN NASAL LAVAGE

5.1 Introduction

Histamine has been shown to induce both vascular permeability and glandular secretion. The effect of histamine on vascular responses is a direct action of histamine on its receptors, located on the vessels. *In vitro*, histamine failed to induce glandular secretion from human nasal mucosal explants (Mullol *et al.*, 1992b), and no H₁ histamine receptors have been identified on submucosal glands, thus histamine-induced glandular secretion appears to be an indirect effect. It was proposed that histamine induced glandular secretion via activation of neuronal reflexes (Raphael *et al.*, 1989b; Baraniuk *et al.*, 1991a; Mullol *et al.*, 1992b)

Human nasal mucosa is richly innervated by the sensory, parasympathetic and sympathetic nervous systems. The involvement of the nervous system in allergic rhinitis has been documented (Sanico *et al.*, 1998). Nasonasal reflex is a term that is now largely used for a nasal neuronal reflex in which unilateral stimulation of the nasal cavity produces bilateral nasal responses. The response on the non-challenged (contralateral) side is believed to be due to a central nasonasal reflex. This reflex can be induced by nasal provocation with cold dry air (Phillip *et al.*, 1993, Jankowshi *et al.*, 1993a) and oral provocation with chilli pepper (Raphael *et al.*, 1989a).

Unilateral cold dry air nasal challenge in subjects with reactivity to cold dry air, produced bilateral nasal secretion which was inhibited by pretreatment with lidocaine on the challenged (ipsilateral) side (Phillip *et al.*, 1993). This supports the importance of neuronal mechanisms in airway responsiveness to an environmental stimulus. In

subjects with allergic rhinitis, unilateral challenge with allergen led to an increase in ipsilateral and contralateral secretion weights (Wagenmann *et al.*, 1994b; Baroody *et al.*, 1994). In this chapter, the effect of unilateral nasal challenge with histamine on secretory responses and the protein content in nasal lavage, in both nasal cavities, was examined in normal subjects.

5.2 Experimental protocol

For the following experiments, the study of nasal secretory activity was conducted on separate occasions from the study of the proteins released. Normal, healthy volunteers aged 20 to 40 years were used. No subject was taking any medication at the time of the experiments and in the week prior to the study. A double-blind, cross-over design was used in all of the protocols. Each subject underwent unilateral challenge with histamine. The same concentrations of histamine as in previous studies were used. Each subject received all of the doses of histamine and saline in random order and on separate days.

5.2.1 Nasal secretion

The amount of nasal secretion was measured as previously described. Three minutes after the baseline measurement, histamine or saline was delivered into one nostril by a handheld pump spray. Nasal secretions were collected from both nostrils 1 minute after the challenge. Then, further measurements were taken every 3 minutes. The sequence of the protocol is shown in figure 5.1.

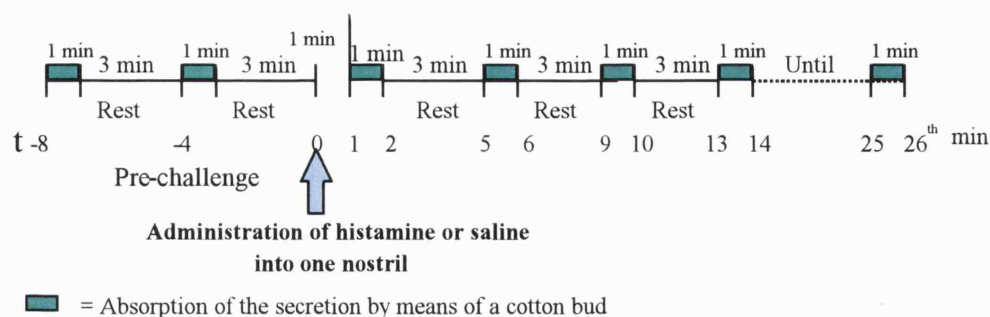


Figure 5.1: Protocol for investigating the effect of unilateral histamine challenge on nasal secretion of both nostrils.

5.2.2 Nasal lavage

Nasal lavages were performed by using a 10 ml syringe as previously described in chapter 2. However, a slight modification was employed. While a lavage was carried out in one nostril, a thumb was pressing on the opposite nostril to prevent lavage fluid from entering the opposite nostril. Figure 5.2 illustrates a test subject performing nasal lavage.

Three nasal lavages were performed prior to histamine challenge. The third lavage from each nostril was kept separately for protein assay. Ten minutes after administration of histamine, a nasal lavage was carried out on each nostril. The lavage from each nostril was kept separately for protein assay. The sequence of the lavage protocol is shown in figure 5.3.

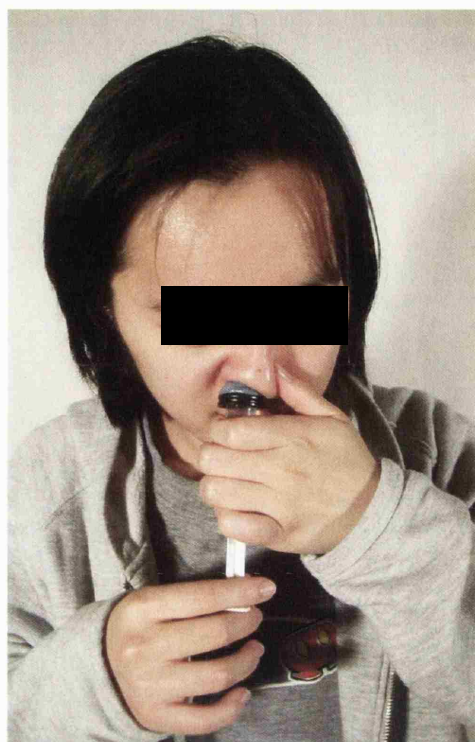


Figure 5.2: Subject undergoing nasal lavage

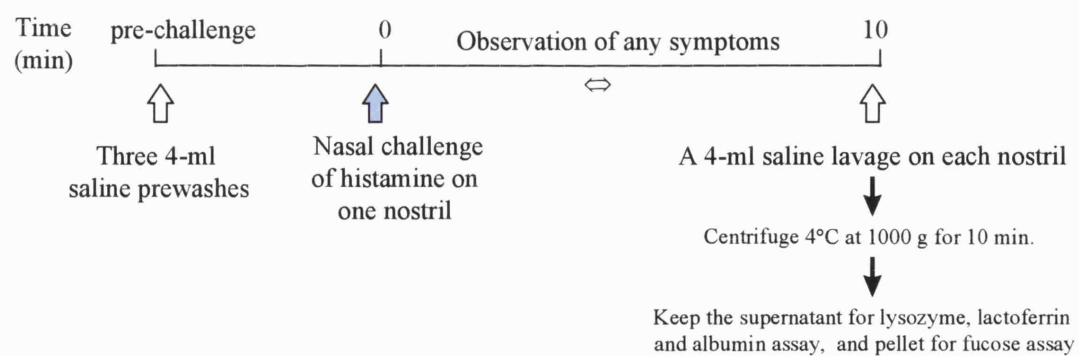


Figure 5.3: Protocol for investigating the effect of unilateral histamine challenge on protein content in nasal lavage.

5.2.3 Record of symptoms

Number of sneezes was counted and recorded after every nasal challenge. Any other symptoms, such as itching, pain and sore throat, experienced were given voluntarily by the subjects and noted.

5.2.4 Data analysis

The medians and percentiles were determined for all parameters: the rate of nasal secretion (mg/min), the area under the curve (AUC) of secretion-response over 26 minutes of observation and the protein concentration in nasal lavage. A Friedman's test was performed to compare the effects of a series of increasing doses of challenge solutions on a group of individuals. Dunn's test was performed following the Friedman's test when there was a statistically significant difference in the treatments, in order to determine exactly which treatment has an effect in comparison to the saline control. This test was performed on the secretory response at each time point, on the AUC and on the protein content of nasal lavage. A p-value ≤ 0.05 was considered to be significant.

5.3 Results

5.3.1 Effect of one nostril challenge with histamine on nasal secretion.

Eight subjects were challenged with saline and histamine, at doses of 100, 300 and 1000 μg , in one nostril. Sneezing and itching were observed following histamine provocation in all subjects, table 5.1 shows the median number of sneezes. No other symptoms were observed. On the ipsilateral side, an increase in nasal secretion was observed from 2 minutes to 10 minutes following histamine challenges (Friedman's test, p-values < 0.02), figure 5.4 (A). Histamine 1000 μg induced a significant increase

in nasal secretion from that of saline at $t = 2$ minutes (Dunn's test, $p\text{-value} < 0.05$). The increase in nasal secretion at $t = 6$ minutes, by all three doses of histamine, was significantly greater than that of saline (Dunn's test, $p\text{-values} < 0.05$). At 10 minutes, 300 and 1000 μg histamine, but not 100 μg histamine, induced a significant increase in nasal secretion compared with that of saline (Dunn's test, $p\text{-values} < 0.05$). There were significant increases in nasal secretion on the contralateral side, in response to ipsilateral stimulation (Friedman's test, $p\text{-values} < 0.05$). Ipsilateral stimulation of 1000 μg histamine induced a significant increase in contralateral secretion at 6 minutes (Dunn's test, $p\text{-value} < 0.05$), while ipsilateral stimulation of 300 μg histamine induced a significant increase in contralateral secretion at 10 minutes (Dunn's test, $p\text{-value} < 0.05$), figure 5.4 (B).

Table 5.1: The median number of sneezes following unilateral histamine challenge in 8 subjects.

Histamine dose	Median number of sneezes	25% percentile	75% percentile
100 μg	1	0	2
300 μg	2	1	4
1000 μg	3	2	5

Figure 5.5 shows the secretory responses over a 26-minute period of observation, reported as AUC. There was an ipsilateral increase in AUC (Friedman's test, $p\text{-values} \leq 0.01$). The ipsilateral responses to histamine were significantly higher than those for saline, following 300 and 1000 μg histamine (Dunn's test, $p\text{-values} < 0.05$). For the contralateral nasal response, the increase in secretion over the 26-minute period was not significantly different from that of saline (Friedman's test, $p\text{-value} > 0.5$).

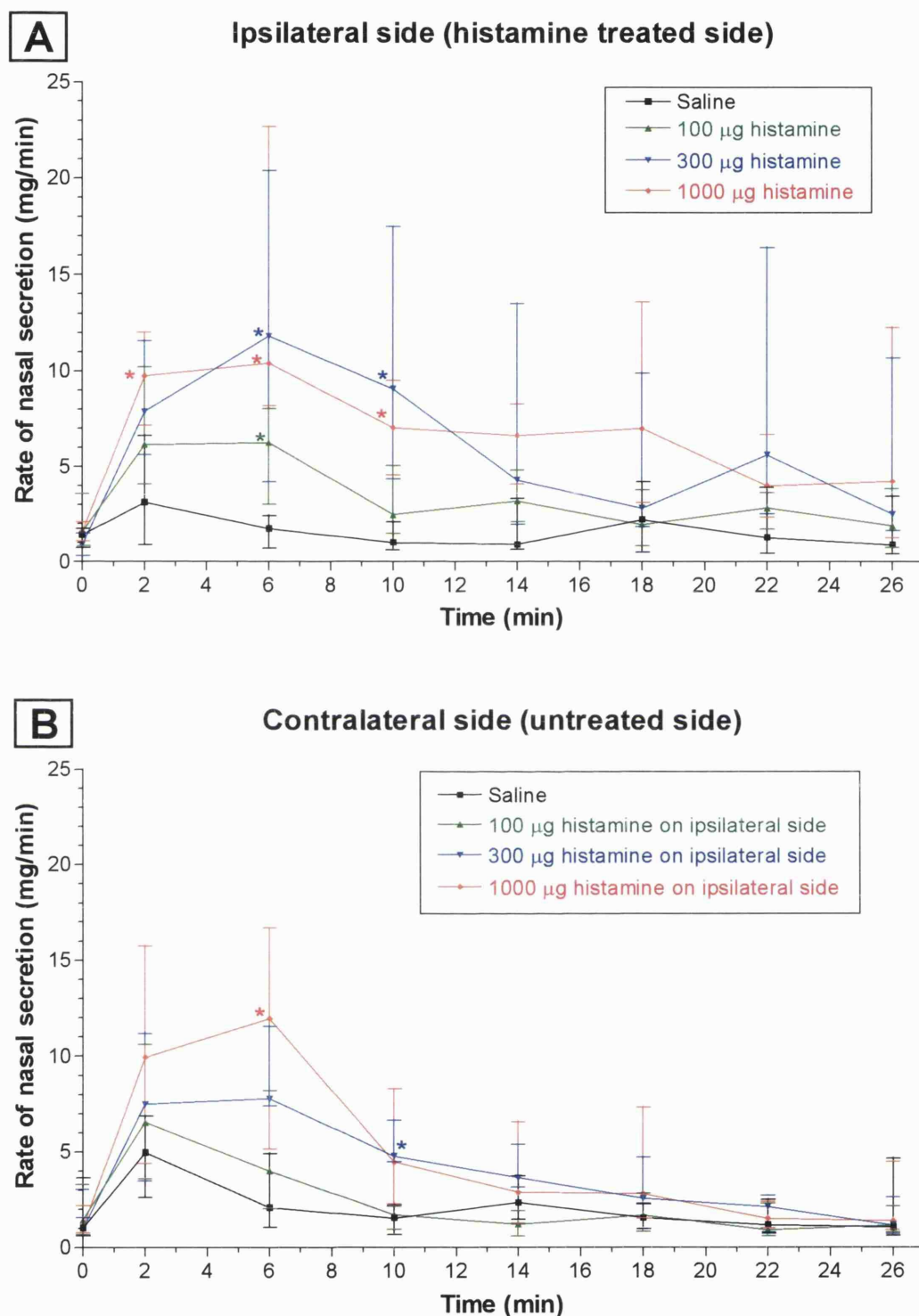


Figure 5.4 (A) and (B): The duration of effect of unilateral histamine challenge over 26 minutes is presented here. (A) shows secretory responses on the ipsilateral side and (B) shows secretory responses on the contralateral side. The data are the medians of 8 subjects with the 25th and 75th percentile values. A statistically significant difference from the saline value at each time point is indicated by * (* p-value < 0.05).

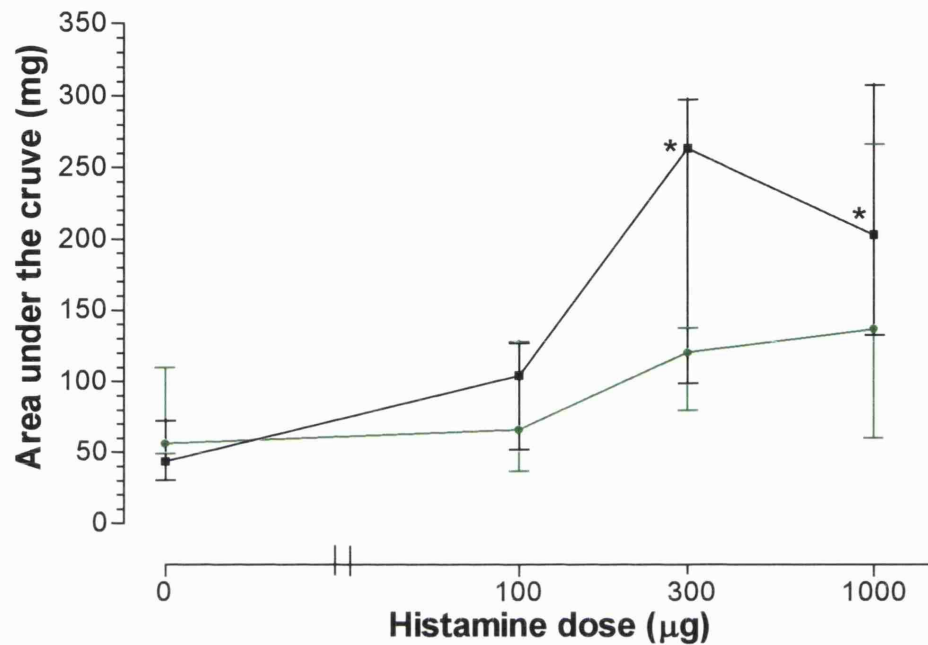


Figure 5.5: Dose-response relationship of nasal secretion to increasing dosage of histamine (challenged side, black line, and contralateral side, green line) are presented here. The dose of histamine was plotted on a \log_{10} scale against the secretory response over 26-minute periods. The data are the medians of 8 subjects with the 25th and 75th percentile values. A statistically significant difference in the secretory response from that of saline is indicated by * (*p-value < 0.05). Unilateral histamine challenges induced an increase in ipsilateral nasal secretion (Friedman's test, p-value \leq 0.01).

5.3.2 Effect of one nostril challenge with histamine on protein content in nasal lavage.

Eleven subjects underwent this study. Sneezing and itching were observed following 300 and 1000 µg histamine provocation in all subjects, table 5.2. Nasal blockage was reported in all subjects on the ipsilateral side after 300 and 1000 µg histamine. No other symptoms were observed. Unilateral treatment with histamine induced an increase in lactoferrin level compared with that of saline in the ipsilateral nostril (Friedman's test, p -values < 0.04), figure 5.6 black line. The increase in lactoferrin was significant following 1000 µg histamine (Dunn's test, p -value < 0.05). There was no significant increase in the lactoferrin level on the contralateral side (Friedman's test, p -value > 0.05), figure 5.6 green line.

Table 5.2: The median number of sneezes following unilateral histamine challenge in 11 subjects.

Histamine dose	Median number of sneezes	25% percentile	75% percentile
100 µg	0	0	1
300 µg	1	0	2
1000 µg	2	0.2	4

On the ipsilateral side, after nasal provocation with histamine, there was a significant increase in lysozyme concentration in the nasal lavage (Friedman's test, p -values < 0.04), with 1000µg histamine inducing a significant increase in the response compared with saline (Dunn's test, p -value < 0.05), figure 5.7 black line. On the contralateral side, there was no significant increase in lysozyme after ipsilateral histamine provocation (Friedman's test, p -values > 0.05), figure 5.7 green line.

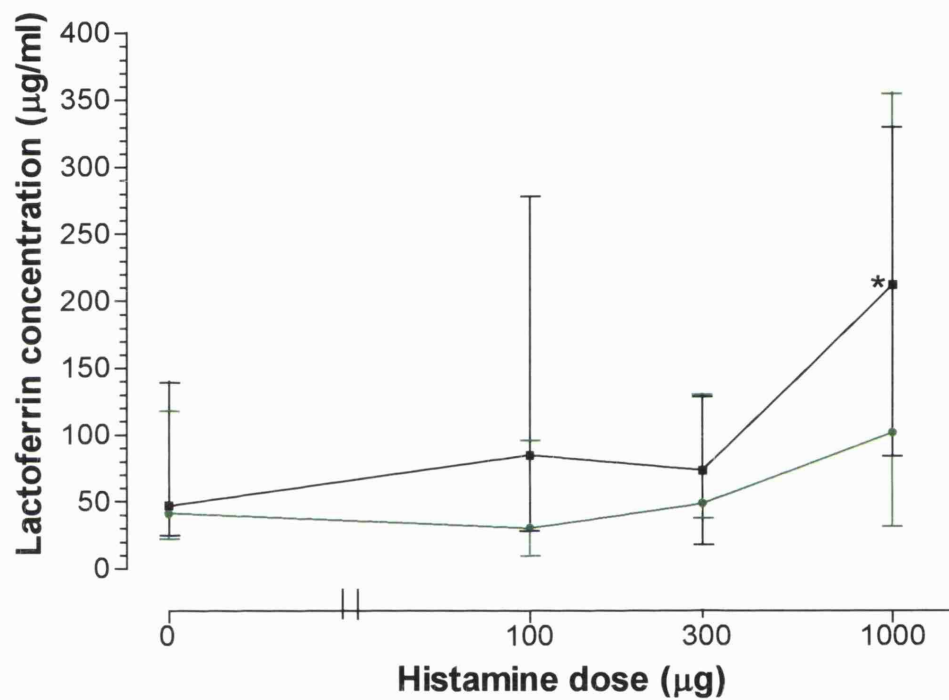


Figure 5.6: Dose-responses of lactoferrin content to increasing doses of histamine (challenged side, black line, and contralateral side, green line) are presented here. The dose of histamine was plotted on a log₁₀ scale against the concentration of lactoferrin. The data are the medians of 11 subjects with the 25th and 75th percentile values. A statistically significant difference in the secretory response from that of saline is indicated by * (* p-value < 0.05). Histamine induced an increase in lactoferrin concentration on the ipsilateral side (Friedman's test, p-value < 0.04) but not on the contralateral side (Friedman's test, p-value > 0.05).

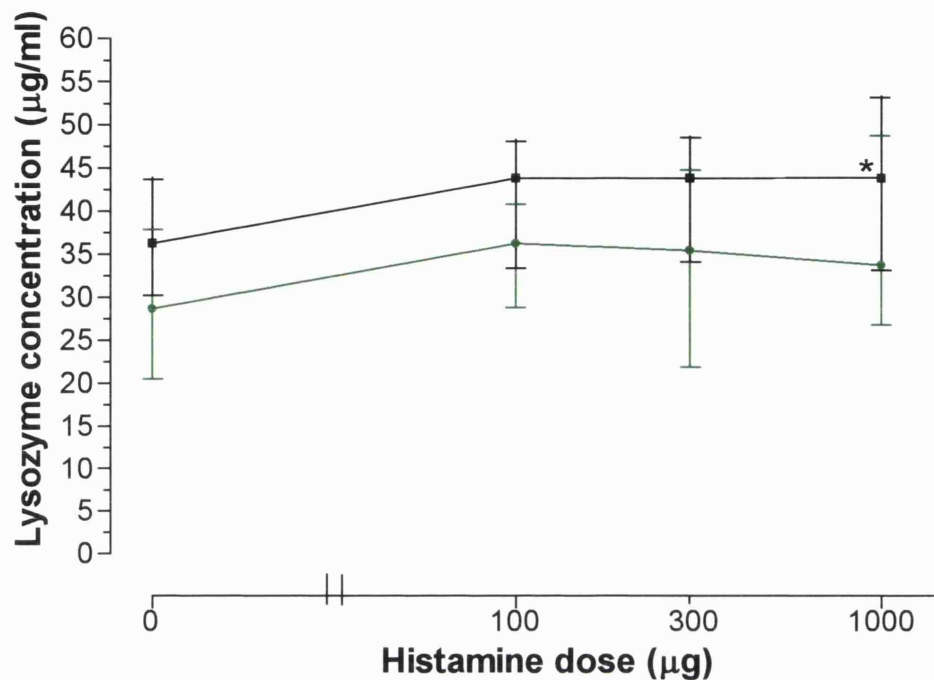


Figure 5.7: Dose-response relationship of lysozyme content in nasal lavage to histamine challenges (challenged side, black line, and contralateral side, green line). The dose of histamine was plotted on a \log_{10} scale against the concentration of lysozyme. The data are the medians of 11 subjects with the 25th and 75th percentile values. Any statistically significant difference in secretory response from that of the saline is indicated by * (*p-value < 0.05). There was an increase in lysozyme concentration on the ipsilateral side following unilateral histamine challenges (Friedman's test, p-values < 0.05).

On both sides, there were increases in albumin content of the nasal lavage after administration of histamine (Friedman's test, p-value ≤ 0.001 for the ipsilateral side and p-value < 0.04 for the contralateral side), figure 5.8. All three doses of histamine induced a significant increase in albumin in the ipsilateral side (Dunn's test with respect to the saline value, p-values < 0.05). A significant increase in the albumin content on the contralateral side was seen in response to ipsilateral stimulation with 300 and 1000 µg histamine (Dunn's test with respect to the saline value, p-values < 0.05).

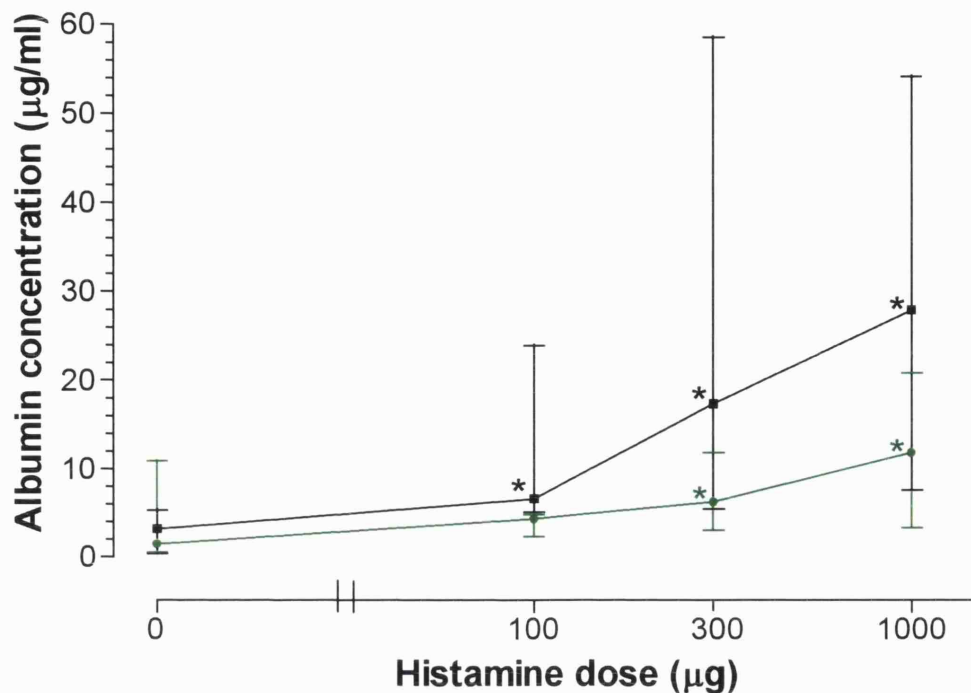


Figure 5.8: Dose-response relationship of albumin content to increasing dose of histamine (challenged side, black line, and contralateral side, green line). The dose of histamine was plotted on a \log_{10} scale against the concentration of albumin. The data are the medians of 11 subjects with the 25th and 75th percentile values. A statistically significant difference in the secretory response from that of saline is indicated by * and * (* and * p-value < 0.05). Histamine induced an increase in albumin concentration on both sides (Friedman's test, p-values < 0.05).

Histamine induced an increase in fucose concentration on the ipsilateral side (Friedman's test, p-values ≤ 0.001) but not in the contralateral side (Friedman's test, p-values > 0.09), figure 5.9. On the ipsilateral side, the significant response occurred following 300 and 1000 µg histamine (Dunn's test with respect to saline value, p-values < 0.05).

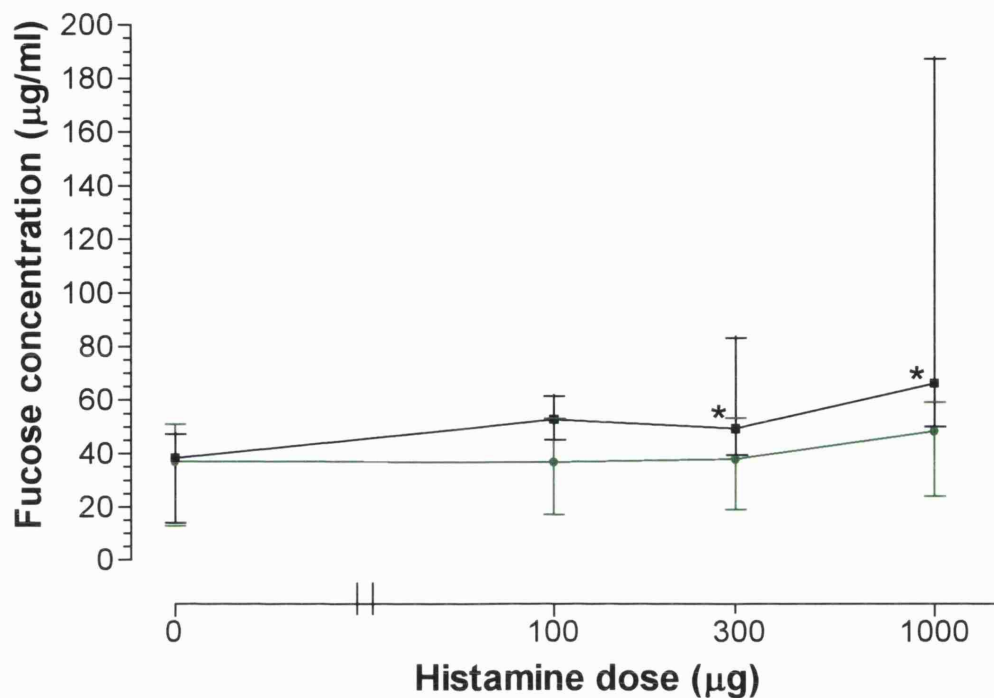


Figure 5.9: Dose-response relationship of fucose content to increasing doses of histamine (challenged side, black line, and contralateral side, green line). The dose of histamine was plotted on a \log_{10} scale against the concentration of fucose. The data are the medians of 11 subjects with the 25th and 75th percentile values. Any statistically significant difference in the secretory response from that of saline will be indicated by * (* p-value < 0.05). Histamine induced an increase in fucose concentration on the ipsilateral side (Friedman's test, p-value \leq 0.001) but not on the contralateral side (Friedman's test, p-value > 0.09).

5.4 Discussion

In this chapter, a single nostril challenge with increasing doses of histamine was employed in order to observe the response on the ipsilateral and the contralateral (reflex response) sides. Histamine challenge induced an increase in the ipsilateral nasal secretion and in the contralateral secretion. The increase in the contralateral secretion occurred briefly at 6 and 10 minutes after 1000 and 300 μg histamine challenge, respectively. Other studies have demonstrated bilateral secretion after unilateral histamine challenge (Rapheal *et al.*, 1989b; Naclerio & Baroody, 1992b; Baroody *et al.*, 1993). Ipsilateral nasal secretion is a mixture of plasma and glandular protein since albumin, lactoferrin, lysozyme and fucose are significantly increased. The contralateral

secretion observed between at 6 and 10 minutes was of plasma protein since only albumin was significantly increased.

A study performed by Raphael *et al* (1989b) showed that unilateral histamine challenge induced a contralateral secretion that contains albumin and secretory IgA (sIgA), another marker of serous cells, indicating that histamine challenge induced vascular and glandular reflex secretion. Based upon this study, however, unilateral histamine challenge does not induce reflex glandular secretion. It is possible that for unilateral provocation a higher concentration of histamine is needed in order to produce a statistically significant glandular secretion on the contralateral side. On the contralateral side, the vascular reflex response was more susceptible to histamine challenge on the opposite side since only plasma protein (albumin), but not glandular protein, was increased significantly. The histamine-induced reflex secretory response on the contralateral side is inhibited by pretreatment with atropine (Naclerio & Baroody, 1992b; Baroody *et al.*, 1993). The inhibitory effect of atropine on contralateral secretion suggests that the histamine-induced reflex secretory response involved the parasympathetic nervous system. Pretreatment with the H₁ antagonist, terfenadine results in inhibition of both ipsilateral and contralateral secretion (Baroody *et al.*, 1993; Jankowski *et al.*, 1993b). Topical administration of another H₁ antagonist, diphenhydramine, on the ipsilateral nasal cavity led to a significant inhibition of an increase in the amount of ipsilateral and contralateral secretion in response to histamine, but no inhibitory effect was observed when it was applied contralaterally (Baroody *et al.*, 1993). These results suggest that the reflex response to unilateral histamine provocation is mediated partly through activation of H₁ receptors.

Histamine effects on the nasal mucosa have been shown to involve both direct action, via H₁ receptors, and indirect action (Raphael *et al.*, 1989b). Histamine provocation induces not only direct vascular responses but also recruitment of parasympathetic and central reflexes such as sneeze. Sneezing is due to the stimulation of an afferent sensory arm that results in the activity of an efferent effector pathway. The efferent

effector pathway is mediated by a complex neural system, however, one of the effector pathways is the recruitment of a parasympathetic reflex with the release of acetylcholine which, in turn, induces glandular secretion. The afferent arm is made up of primary sensory neurones with cell bodies located in the trigeminal ganglion. The ganglia contain a large variety of sensory neurones that are distinct according to their morphology, velocity and modality of impulse conduction and neurotransmitter pattern. Sneezing had been shown in chapter 4 and also by other studies (Naclerio *et al.*, 1989; Togias *et al.*, 1989; Mygind *et al.*, 1983) to be inhibited by pretreatment with H₁ receptor antagonists. These findings suggest that the central reflex (sneeze) responds to histamine through activation of H₁ receptors.

Activation of the sensory nerves by histamine generates antidromic impulses and an axon reflex, which leads to the release of neuropeptides such as substance P, neurokinin A (NKA) and calcitonin gene-related peptide (CGRP). The neuropeptides released from these neurones have their own unique actions. Nasal provocation with substance P and NKA increased nasal airway resistance in a dose-dependent manner (Devillier *et al.*, 1988; Braunstein *et al.*, 1991). Unilateral histamine challenge may, therefore, induce a reflex vascular response, vasodilatation and increased vascular permeability, through the activation of axon reflexes. Pretreatment with atropine has no effect on contralateral albumin (Raphael *et al.*, 1989b), indicating that the increase in vascular permeability is not due to a parasympathetic reflex.

Stimulation of sensory nerves by histamine and possibly other stimuli may initiate central parasympathetic reflexes. Pretreatment with atropine resulted in an inhibition of the amount of contralateral secretion in response to histamine (Naclerio & Baroody, 1992b; Baroody *et al.*, 1993) and partial inhibition of the ipsilateral histamine response (Baroody *et al.*, 1993). Furthermore, unilateral nasal provocation with bradykinin, induced an increase in contralateral lactoferrin which was inhibited by atropine (Riccio & Proud, 1996). Bradykinin also caused a contralateral increase in total protein and

glycoconjugates which was inhibited by ipratropium bromide (Baraniuk *et al.*, 1994a). These data implicate the role of parasympathetic reflex on contralateral secretion.

Naclerio and Baroody (1992b and 1995a) measured the amount of secretion from both nostrils following unilateral histamine challenge. The protein content in the secretion was not studied. A dose of 300 µg histamine, delivered onto a small surface of nasal mucosa by the use of filter paper discs, stimulates contralateral secretion. Another study of unilateral histamine challenge, investigating the protein content in the contralateral secretion using a single dose of 1000 µg histamine delivered by handheld nebulizer, showed that the contralateral secretion is of both vascular and serous cell origin. However, there was a higher proportion of protein of serous cell origin, indicated by sIgA, than vascular protein (Raphael *et al.*, 1989a). In this chapter 300 and 1000 µg of histamine was needed in order to induce a significant increase in the contralateral secretion which lasted for a brief period of time. The protein content in the contralateral secretion was of vascular origin.

In conclusion, unilateral histamine challenges preferentially stimulated vascular reflex responses as indicated by an increase in contralateral albumin content. The vascular reflex responses are more sensitive than glandular responses since lower concentrations of histamine were enough to induce an increase in albumin on the contralateral side. The vascular reflex may be due to the release of neuropeptides via an axon reflex mechanism. A higher histamine concentration may be needed in order to induce contralateral glandular secretion. When a large volume of a dilute solution of histamine was applied to a greater surface of nasal mucosa by means of a spray, a higher concentration of histamine was needed in order to induce significant contralateral secretion. Lower concentrations of histamine, on the other hand, were able to induce significant contralateral secretion, when histamine solution was applied by the use of filter paper discs. This indicates that the technique of delivery may elicit different types of reflex responses and that glandular reflex response needs a greater magnitude of stimulation.

CHAPTER 6

THE EFFECT OF SUBSTANCE P ON THE NASAL SECRETION AND PROTEIN CONTENT IN NASAL LAVAGE OF NORMAL AND ATOPIC SUBJECTS.

6.1 Introduction

There is growing evidence that substance P is one of the main neuropeptides involved in allergic rhinitis. Substance P levels are significantly increased in nasal lavage immediately after nasal allergen challenge (Mosimann *et al.*, 1993). A dense network of substance P-immunoreactive fibres has been demonstrated in the nasal mucosa (Uddman *et al.*, 1983; Baranuik *et al.*, 1991c; Fang & Shen, 1997). By quantitatively analysing the stained fibres and cells using a graphic Auto CAD program, Fang *et al.* (1998a) showed that allergic rhinitics have a significantly higher tissue concentration of substance P than normal subjects. It was shown that the levels of substance P and vasoactive intestinal peptide (VIP) in nasal secretion from patients suffering from house dust nasal allergy were significantly higher than those of the control group (Shinoda *et al.*, 1997). The substance P concentration in nasal lavage increased in a dose-dependent manner after bradykinin nasal provocation in both allergic and non-allergic subjects (Baumgarten *et al.*, 1997).

Substance P is released from sensory nerves upon mucosal injury, inhalation of irritant, capsaicin and inflammatory mediators such as histamine and bradykinin. Substance P has diverse properties which include vasodilatation and increases in vascular permeability (Laitinen *et al.*, 1987), bronchial smooth muscle contraction (Joos *et al.*, 1987; Crimi *et al.*, 1988), skin mast-cell degranulation (Foreman *et al.*, 1987a) and leukocyte chemotaxis (Wiedermann *et al.*, 1993). *In vitro*, substance P has been shown to stimulate mucus secretion from submucosal glands (Baranuik *et al.*, 1991c; Mullol *et al.*, 1992c).

Nasal provocation with substance P induced a dose-dependent increase in nasal airway resistance in both normal (Devillier *et al.*, 1988; Chatelain *et al.*, 1995) and allergic rhinitic subjects (Devillier *et al.*, 1988; Fajac *et al.*, 1995; Konno *et al.*, 1996). In this chapter the effect of substance P on secretory response and the protein content in the nasal lavage of atopic and non-atopic subjects was examined and compared.

6.2 Experimental protocol

For the following experiments, the study of nasal secretory activity was conducted on a separate occasion from the protein release study. Normal, healthy volunteers aged 20 to 40 years were used. In another study, atopic subjects aged 20 to 30 years were used, 4 subjects with seasonal rhinitis and 3 subjects with perennial rhinitis, as defined by medical history. No subject was taking any medication at the time of the experiments and in the week prior to the study. A double-blind, cross-over design was used in all of the protocols. Each subject underwent nasal challenge with all of the three doses of substance P (10, 30 and 50 µg) and saline in random order and on separate days. The concentrations of substance P used were within the range used in a previous study (Devillier *et al.*, 1988; Lurie *et al.*, 1994).

6.2.1 Nasal secretion and cutaneous facial blood flow.

The amount of nasal secretion was measured as previously described. Three minutes after the baseline measurement, substance P was delivered into both nostrils by a handheld pump spray. The amount of nasal secretion was collected from both nostrils 1 minute after the challenge. Then, further measurements were taken every 3 minutes with 1 minute allowed for absorption of secretion into the cotton bud. Measurement of cutaneous facial blood flow, by means of a laser Doppler flowmeter, was taken at four different time points. The first reading was taken before substance P provocation, in order to measure the resting blood flow. Then three more readings were taken at 2, 5 and 10 minutes after substance P challenge. The laser Doppler flowmeter probe was placed on the cheek and held 1 inch from the base of the nose. All recordings were made at the same site. The sequence of the protocol is shown in figure 6.1.

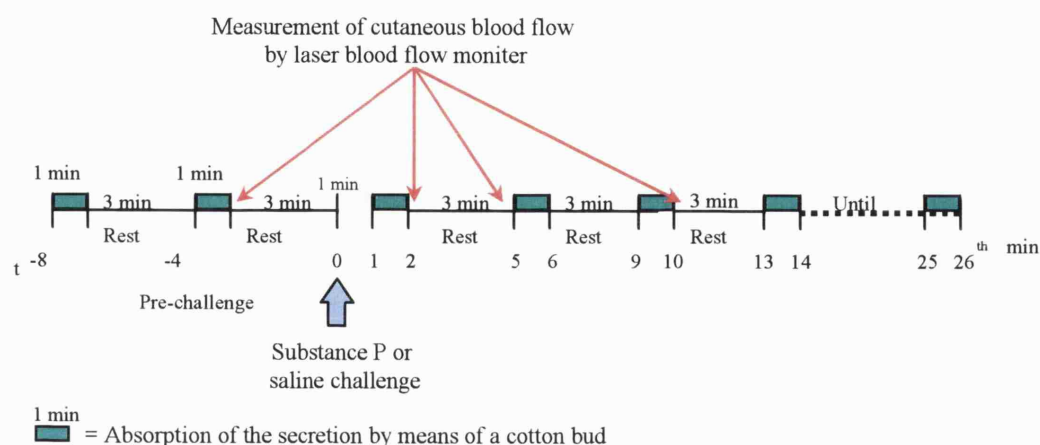


Figure 6.1: Protocol for investigating the effect of substance P on the nasal secretion and cutaneous facial blood flow.

6.2.2 Nasal lavage

Nasal lavages were performed three times per nostril prior to substance P challenge. The third lavage from each nostril was kept for protein assay. Ten minutes after administration of substance P, a nasal lavage was carried out on each nostril. The lavage was kept for protein assay. The sequence of the lavage protocol is shown in figure 6.2.

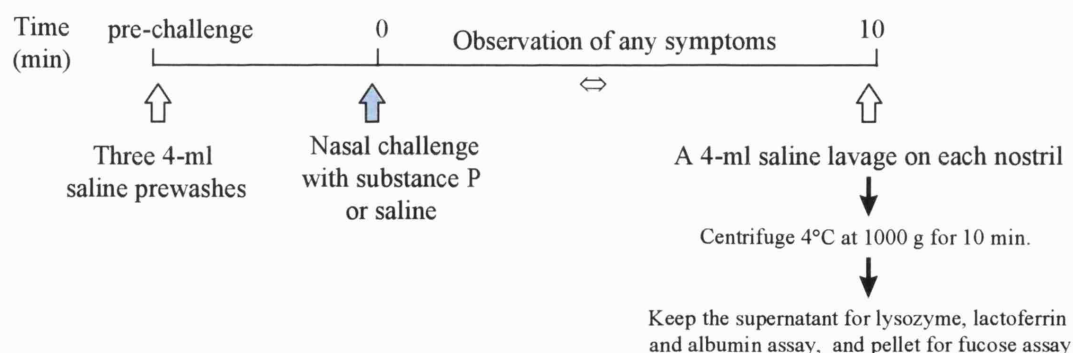


Figure 6.2: Protocol for investigating the effect of substance P on protein content in nasal lavage.

6.2.3 Record of symptoms

If sneezing occurred, the number of sneezes was counted and recorded. Any other symptoms, such as itching, pain and sore throat, experienced were given voluntarily by the subjects and noted.

6.2.4 Data analysis

The medians and percentiles were determined for all parameters: the increase in facial blood flow at 2 minutes; the rate of nasal secretion (mg/min); the area under the curve (AUC) of the secretion response over 26 minutes observation and the protein concentration in nasal lavage. A Friedman's test was performed to compare the effects of a series of increasing doses of challenge solutions on a group of individuals. Dunn's test was performed following the Friedman's test when there was a statistically significant difference in the treatments, in order to determine exactly which treatment has an effect in comparison to saline. A $p\text{-value} \leq 0.05$ was considered to be significant.

For comparison between the responses of normal and atopic subjects, the data was first normalised by calculating the percentage change from saline response. The Mann-Whitney rank sum test was performed to compare the difference between the responses produced by normal and atopic subjects. With Bonferroni's correction factor applied, a $p\text{-value} \leq 0.017$ was considered to be significant.

6.3 Results

6.3.1 Effect of nasal challenge with substance P on nasal secretion of normal subjects.

Eight normal healthy subjects underwent intra-nasal challenge with substance P. Nasal challenge with substance P induced symptoms of warmth, facial flushing and head throbbing, that developed acutely in all subjects. The facial flush faded away

within 6 to 10 minutes. All subjects complained of discomfort (blocked nose and sore throat). Sneezing and pruritus were not observed. Figure 6.3 shows the response of facial cutaneous blood flow after nasal challenge at 2 minutes in 6 subjects. Substance P induced an acute increase in cutaneous blood flow (Friedman's test, p -value < 0.03), which was significant after 50 μg substance P (Dunn's test, p -value < 0.05).

Figure 6.4 (A) shows the duration of the substance P effect on the secretory response. At each time point the increase in nasal secretion, following substance P challenge, was not significantly different from that of saline (Friedman's test, p -values > 0.05). The secretory response, within a 26 minute period of observation, after nasal provocation, with each of the three doses of substance P, showed no significant differences from that of saline provocation (Friedman's test, p -values > 0.4), figure 6.4 (B).

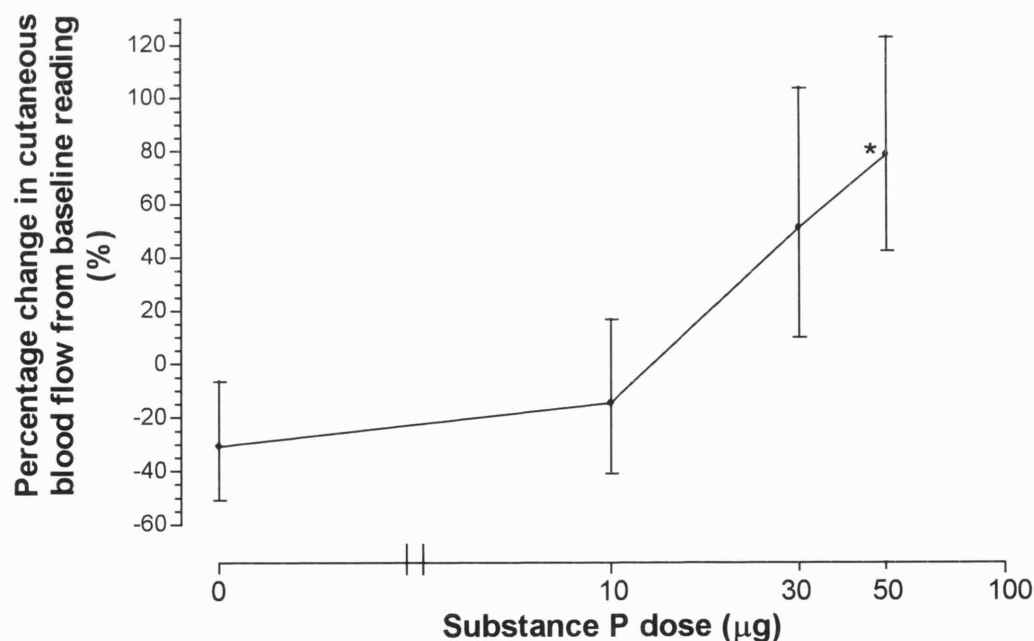


Figure 6.3: The effect of substance P on cutaneous facial blood flow of 6 normal subjects. The data are represented as the median value of the percentage change from baseline with the 25th and 75th percentile values, where the baseline value is 4.7 cm., in 6 subjects. The dose of substance P was plotted on a logarithmic scale. A statistically significant difference from the saline value is indicated by * (* p -value < 0.05). There was an increase in the response after substance P provocation, evaluation by Friedman's test p -value < 0.05 .

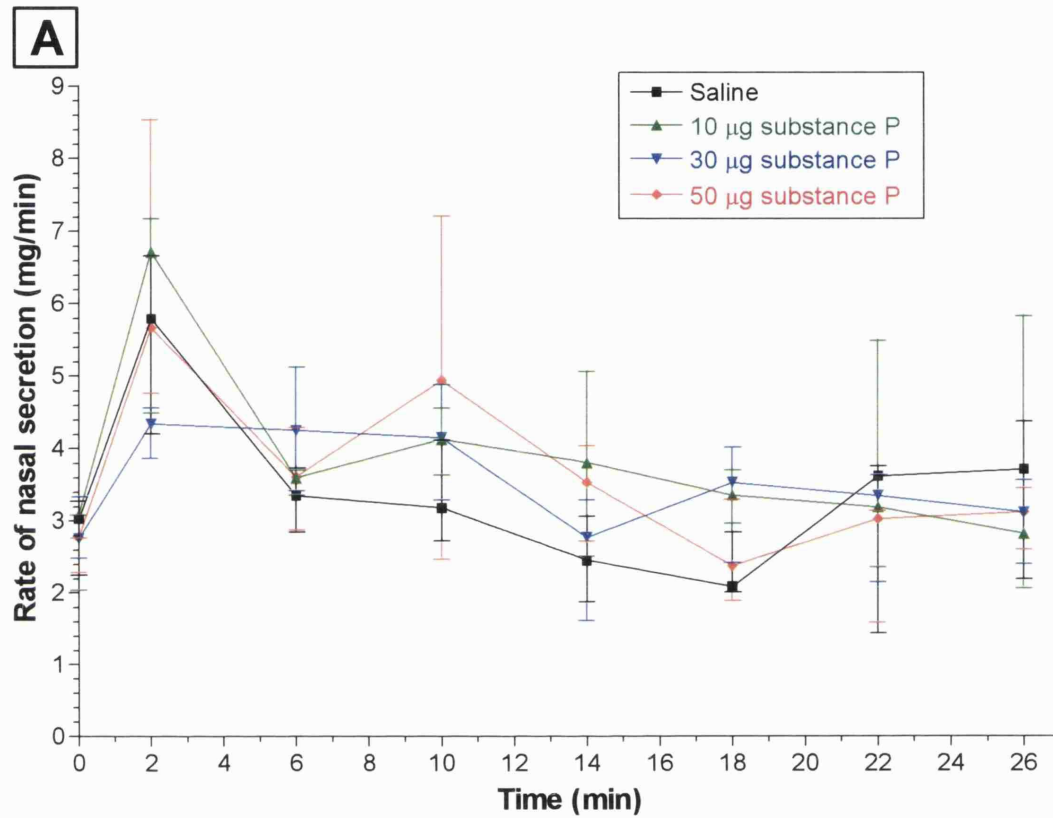


Figure 6.4 (A): The effect of substance P on nasal secretion over a 26-minute period of observation. The data are represented as the medians of 8 normal subjects, with the 25th and 75th percentile values. There was no statistically significant difference from the saline value at each time point.

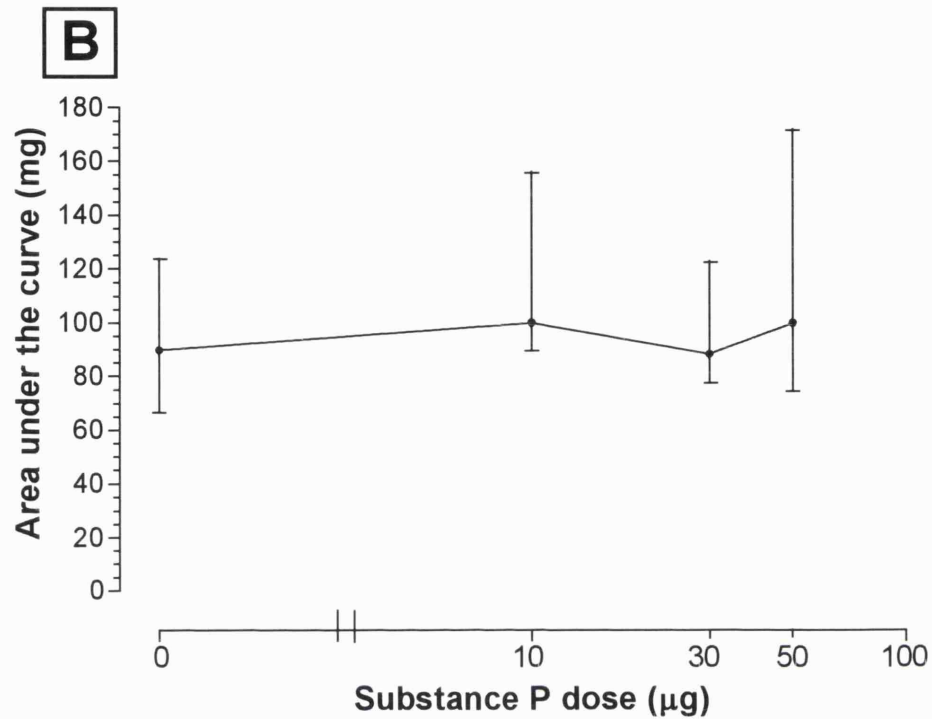


Figure 6.4 (B): Dose-response curve of secretory response to substance P over a 26-minute period of observation is presented. The dose of substance P was plotted on a logarithmic scale. The data are represented as the medians of 8 normal subjects with the 25th and 75th percentile values. There was no increase in secretory response after substance P provocation, evaluation by Friedman's test, p -value > 0.1.

6.3.2 Effect of nasal challenge with substance P on protein content in nasal lavage of normal subjects.

Seven subjects underwent this study. As in the nasal secretory response study, burning, flushing and head pounding were observed after substance P challenge in all subjects. Nasal blockage and flushing from face to neck were observed after 30 and 50 µg of substance P in all subjects. A sore throat and the sensation of having something in the throat were reported in two out of seven subjects. No other nasal symptoms were observed.

There was no significant increase in the lactoferrin, lysozyme and fucose content of nasal lavage (Friedman's test, p-values > 0.1), figure 6.5 (A), (B) and (D). On the other hand, there was a significant increase in albumin content (Friedman's test, p-value < 0.01) with 10 and 50 µg substance P inducing a significant increase in albumin concentration in the nasal lavage from that of saline (Dunn's test, p-values < 0.05), figure 6.5 (C).

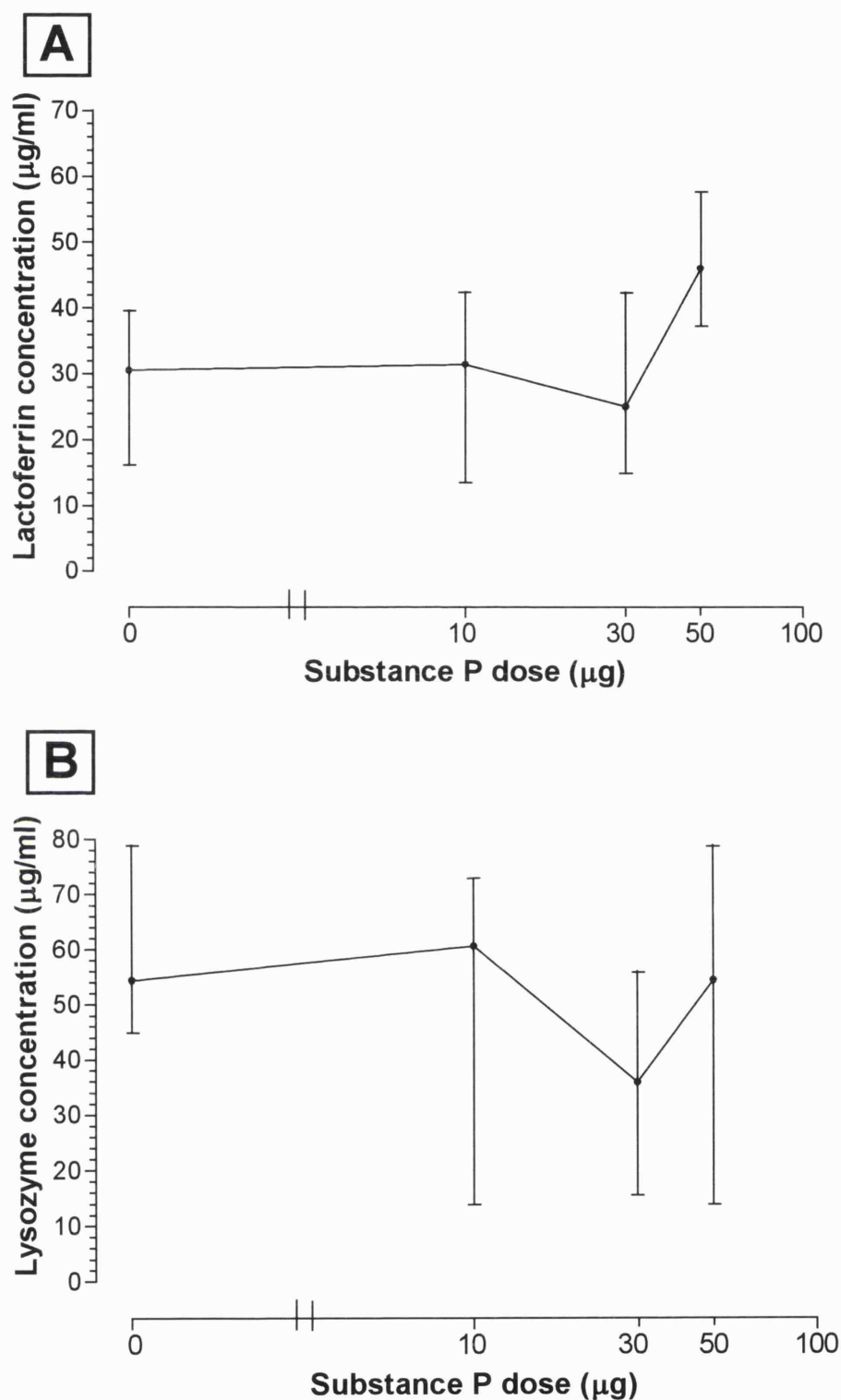


Figure 6.5 (A) and (B): The effect of substance P on protein concentration in nasal lavage of 7 normal subjects. The concentration of lactoferrin (A) and lysozyme (B) were plotted against a logarithmic scale of substance P dose. The concentration of protein is expressed as a median value with the 25th and 75th percentile values. Any statistically significant difference from the saline value will be indicated by * (* p-value < 0.05).

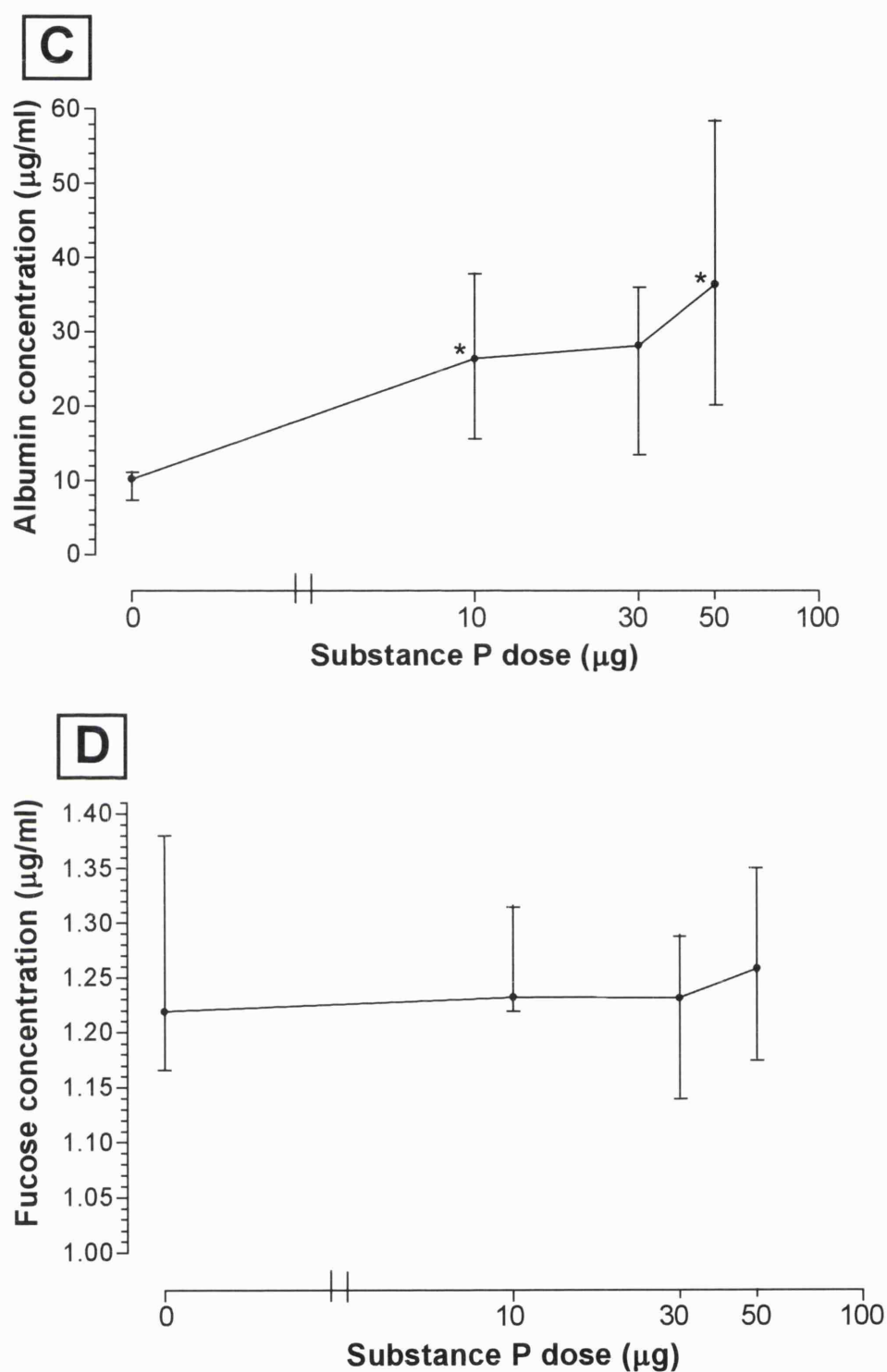


Figure 6.5 (C) and (D): The effect of substance P on protein concentration in nasal lavage of 7 normal subjects. The concentration of albumin (C) and fucose (D) was plotted against a logarithmic scale of substance P dose. The concentration of protein is expressed as a median with the 25th and 75th percentile values. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05). Only the albumin response increased following substance P challenges (Friedman's test, p-value < 0.01).

6.3.3 Effect of nasal challenge with substance P on nasal secretion of atopic subjects.

Substance P nasal challenge in seven atopic subjects induced symptoms of burning and facial flushing that developed acutely in all subjects. Facial flush faded away within 3 to 5 minutes. Following provocation with 50 µg substance P, the flushing was observed from the face down to the neck in all subjects. Sneezing and pruritus were also observed in three subjects. The facial cutaneous blood flow after nasal challenge at 2 minutes is represented in figure 6.6. Substance P induced an increase in facial blood flow (Friedman's test, $p\text{-value} < 0.01$), which was significant after 50 µg (Dunn's test, $p\text{-value} < 0.05$).

Substance P induced an increase in nasal secretion in atopic subjects: in two subjects a pale yellow secretion was observed after 50 µg substance P. However, the increase in nasal secretion at each time point was not significantly different from that of saline provocation (Friedman's test, $p\text{-values} > 0.05$), figure 6.7 (A). The secretory response, over a 26-minute period of observation, after substance P showed no significant difference from that of saline (Friedman's test, $p\text{-value} > 0.2$), figure 6.7 (B).

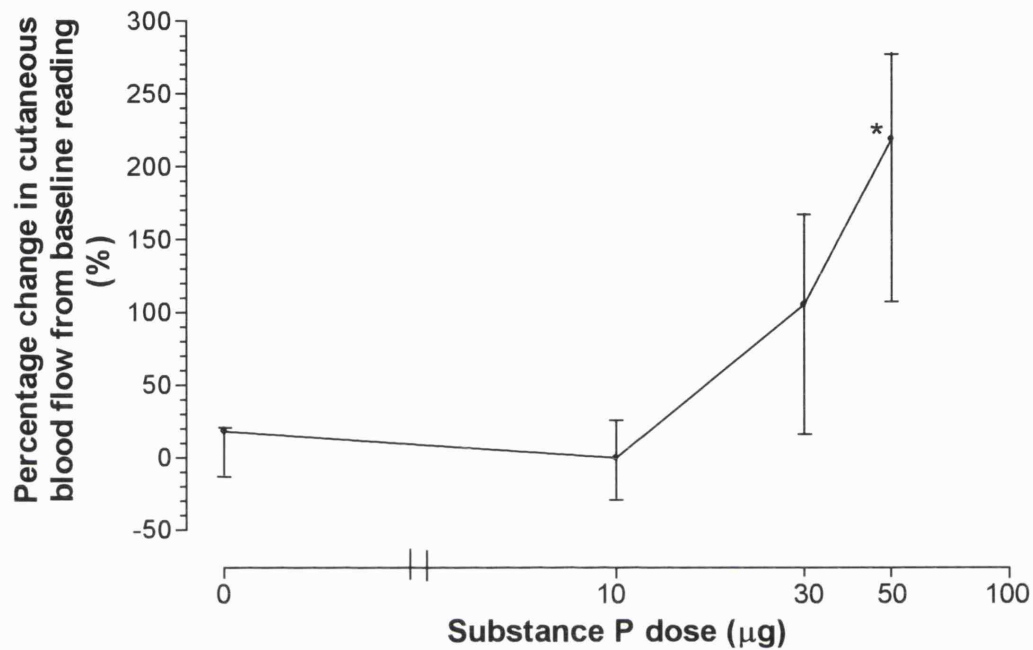


Figure 6.6: The effect of substance P on cutaneous facial blood flow of 7 atopic subjects. The data are represented as the median value of the percentage change from baseline with the 25th and 75th percentile values, where the baseline value is 4.1 cm., in 7 subjects. The dose of substance P was plotted on a logarithmic scale. A statistically significant difference from the saline value is indicated by * (* p-value < 0.05). There was an increase in the response after substance P provocation, evaluation by Friedman's test, p-value < 0.01.

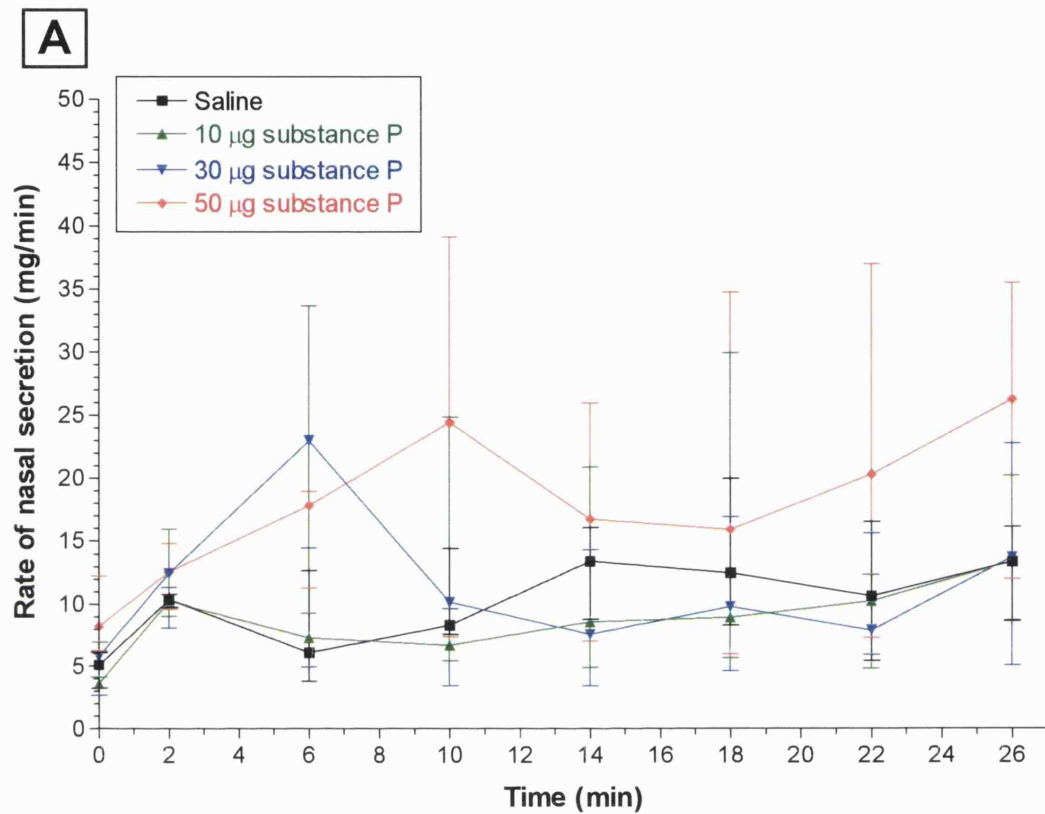


Figure 6.7 (A): The effect of substance P on nasal secretion, over 26 minutes of observation, of 7 atopic subjects. The data are represented as the medians with the 25th and 75th percentile values. There was no significant difference in secretion from that in response to saline at each time point.

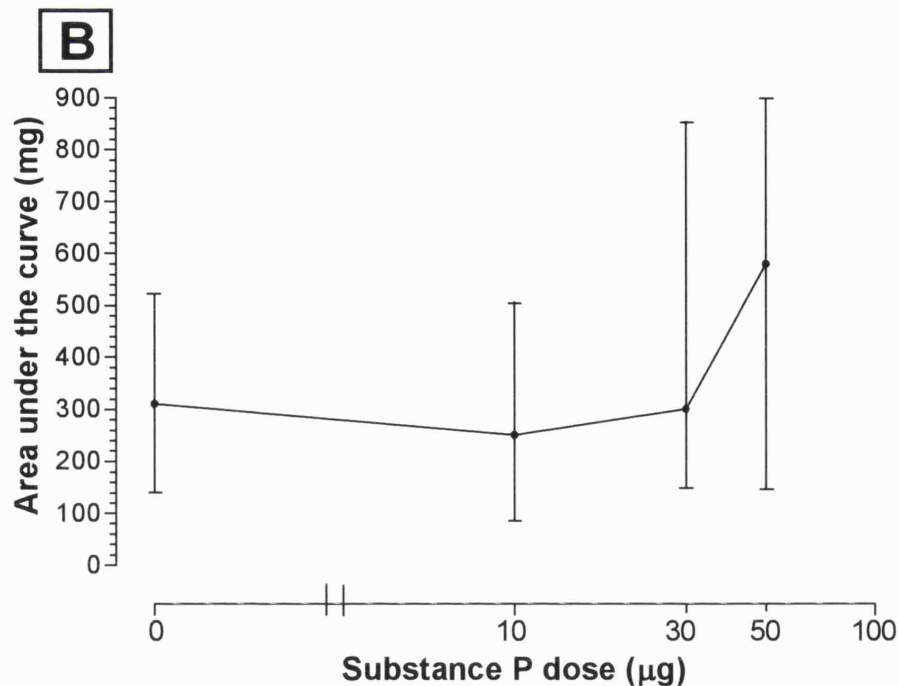


Figure 6.7 (B): Dose-response curve of the secretory response over a 26-minute period of observation is presented. The dose of substance P was plotted on a logarithmic scale. The data are represented as the medians of seven subjects with the 25th and 75th percentile values. A statistically significant difference from the saline response is indicated by * (* p-value < 0.05). There was no increase in secretory response after substance P provocation, evaluation by Friedman's test, p-value > 0.05.

6.3.4 Effect of nasal challenge with substance P on protein content in nasal lavage of atopic subjects.

Seven subjects underwent this study. The same symptoms as in the secretory response study were observed in all subjects. Comparison of changes from the saline value for lactoferrin, lysozyme and fucose after the three doses of substance P, shows that there was no significant increase in these proteins (Friedman's test, p-values > 0.2), figure 6.8 (A), (B) and (D). Albumin content, on the other hand, was significantly increased following substance P challenges (Friedman's test, p-value < 0.01), with all three doses of substance P inducing a significant increase in response from the saline value (Dunn's test, p-values < 0.05), figure 6.8 (C).

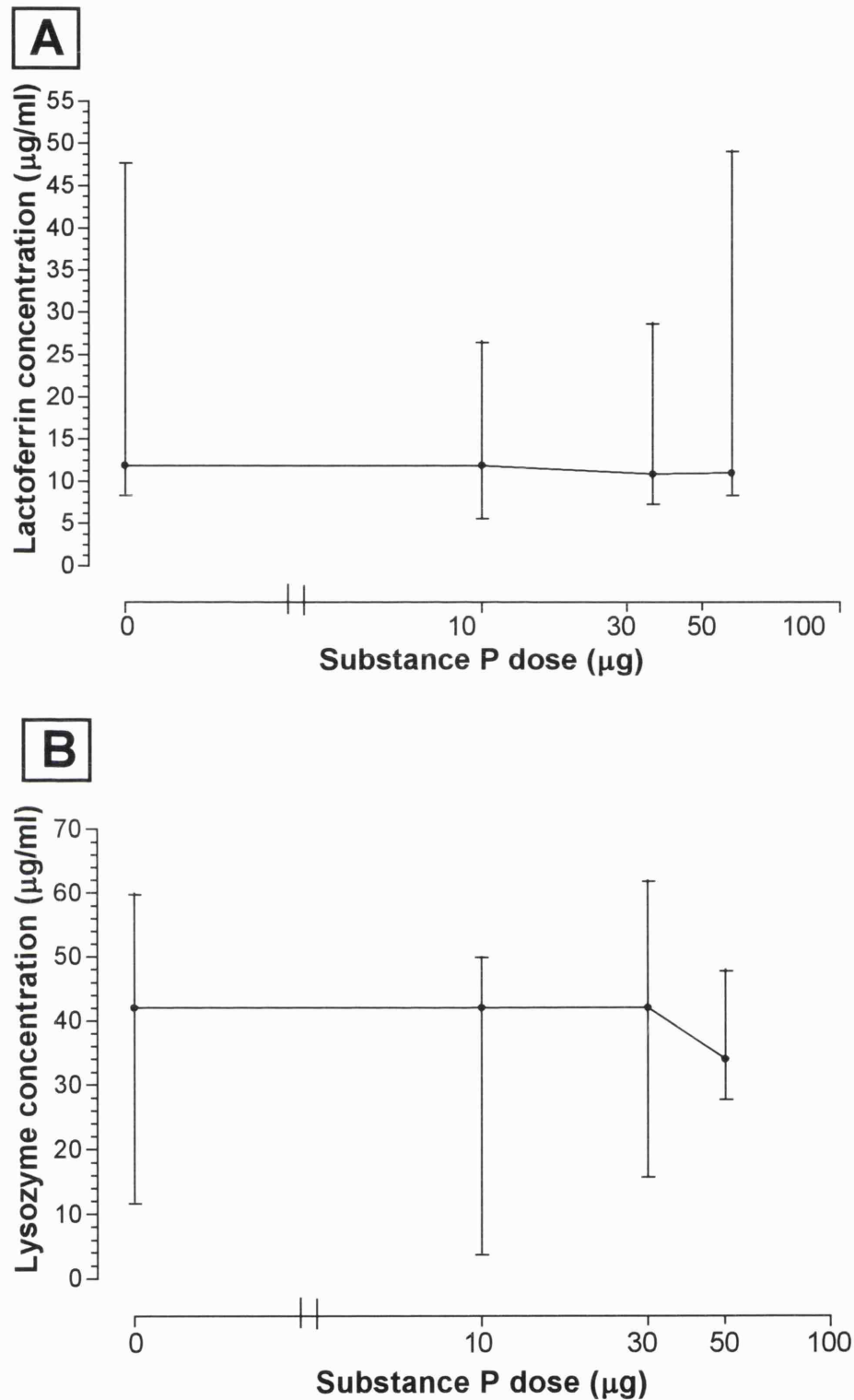


Figure 6.8 (A) and (B): The effect of substance P on lactoferrin (A) and lysozyme (B) concentrations in nasal lavage of 7 atopic subjects. The concentration of lactoferrin (A) and lysozyme (B) were plotted against a logarithmic scale of substance P dose. The concentration of protein is expressed as the medians of seven subjects with the 25th and 75th percentile values. Any statistically significant difference from the saline value will be indicated by * (* p-value < 0.05).

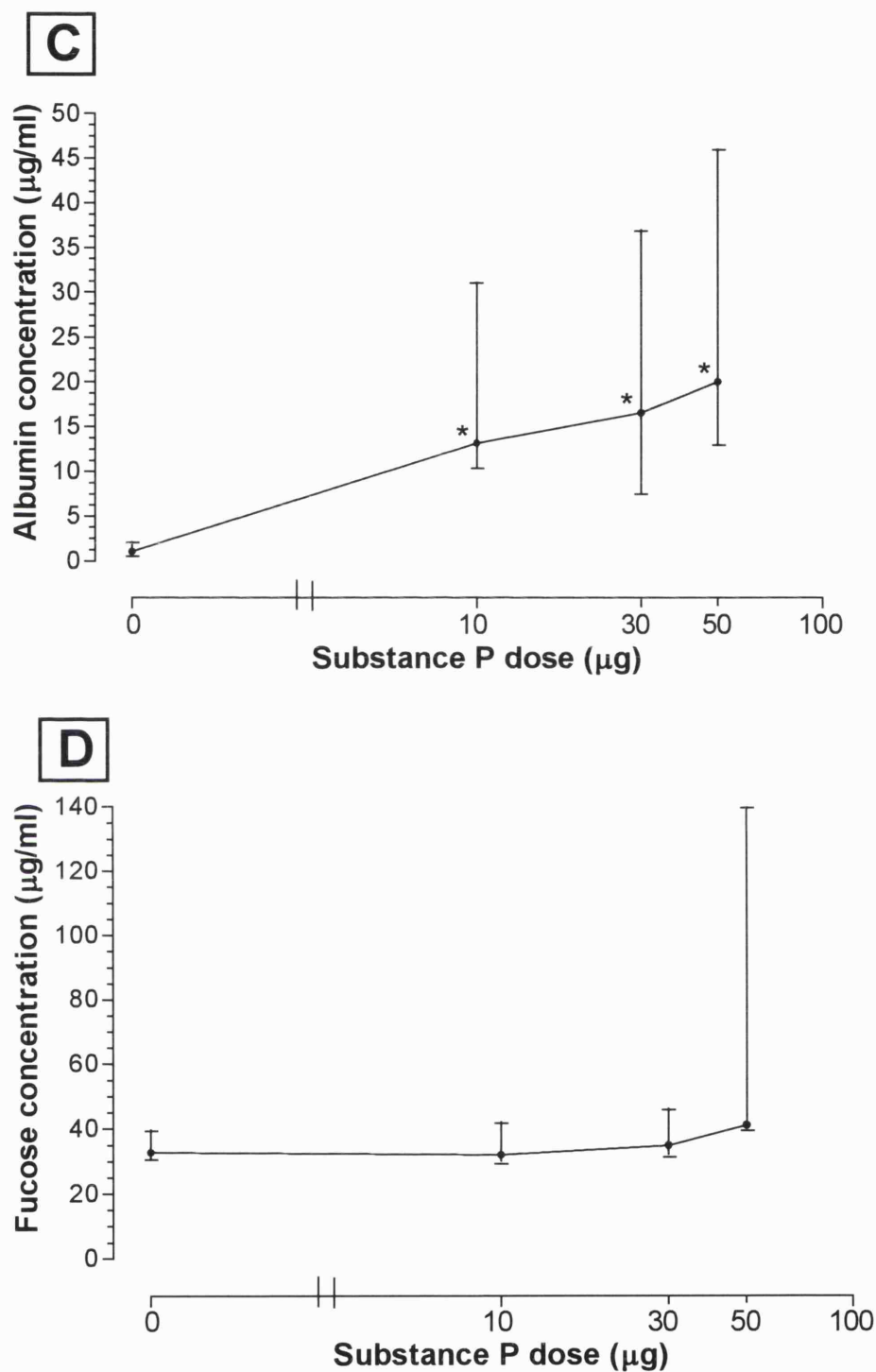


Figure 6.8 (C) and (D): The effect of substance P on albumin (C) and fucose (D) concentrations in nasal lavage of 7 atopic subjects. The concentration of albumin (C) and fucose (D) was plotted against a logarithmic scale of substance P dose. The concentration of protein is expressed as the median of seven subjects with the 25th and 75th percentile values. A statistically significant difference from the saline value is indicated by * (* p -value < 0.05). Only the albumin response is increased following substance P challenges (Friedman's test, p -value < 0.01).

6.3.5 Comparison of responses induced by substance P between normal and atopic subjects.

The data were normalised and expressed as a fold-increase over the effect of saline, due to the variability in the saline (control) response in the two groups. Atopic subjects appeared to produce a greater facial cutaneous blood flow response when compared with normal, non-atopic subjects (figure 6.9). The cutaneous blood flow response after substance P shows a higher response in atopic, than in normal subjects. However the differences between the two groups were not greater than would be expected by chance (Mann-Whitney test with Bonferroni's correction factor, p-values > 0.017). Substance P had no effect on glandular proteins and no significant differences in glandular proteins were observed between atopic and normal subjects (Mann-Whitney test with Bonferroni's correction factor, p-values > 0.017). In contrast, substance P induced an increase in albumin in both atopic and normal subjects. Although the median values of albumin response in both groups were similar, there was marked variation in the saline controls. However, the magnitude of albumin responses compared with that of saline in each individual after all three doses was significantly higher in atopic, than in normal subjects, figure 6.10 (Mann-Whitney test with Bonferroni's correction factor, p-values < 0.017 for all three doses).

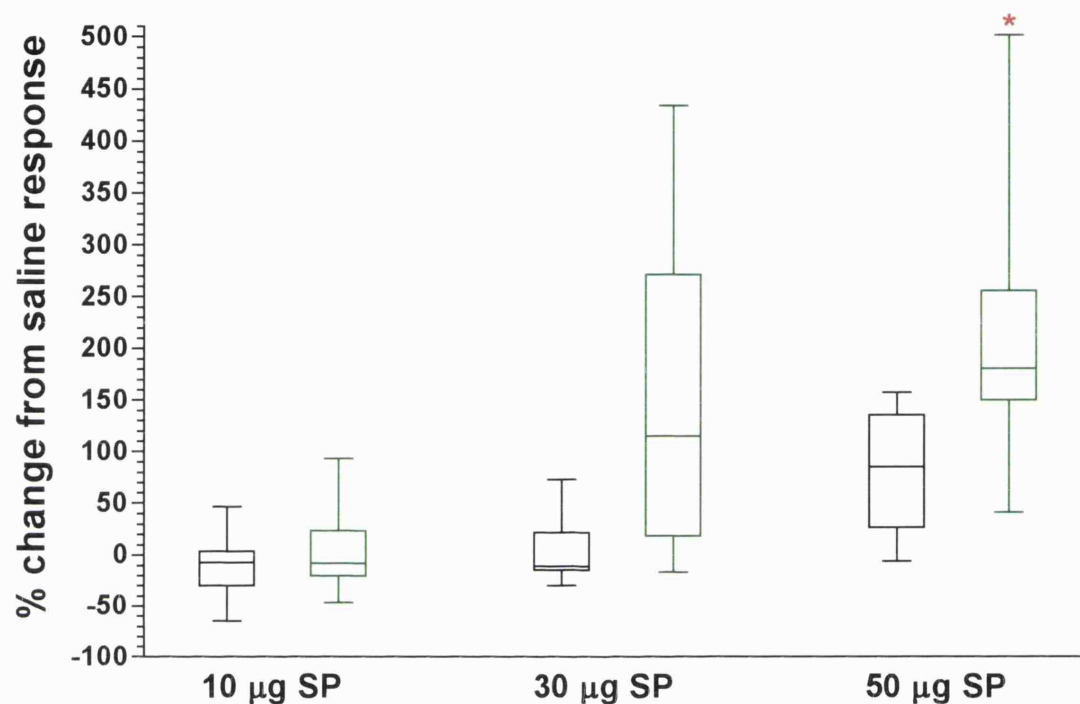


Figure 6.9: The facial cutaneous blood flow responses of both groups (black box for normal, n=6 and green for atopic, n=7) after substance P provocation are presented here. The data have been normalised and are expressed as percentage change from the saline response. The normalised data are presented as box and whiskers plots to compare the effect of substance P in both groups. A statistically significant difference between the two groups is indicated by * (* p-value < 0.05).

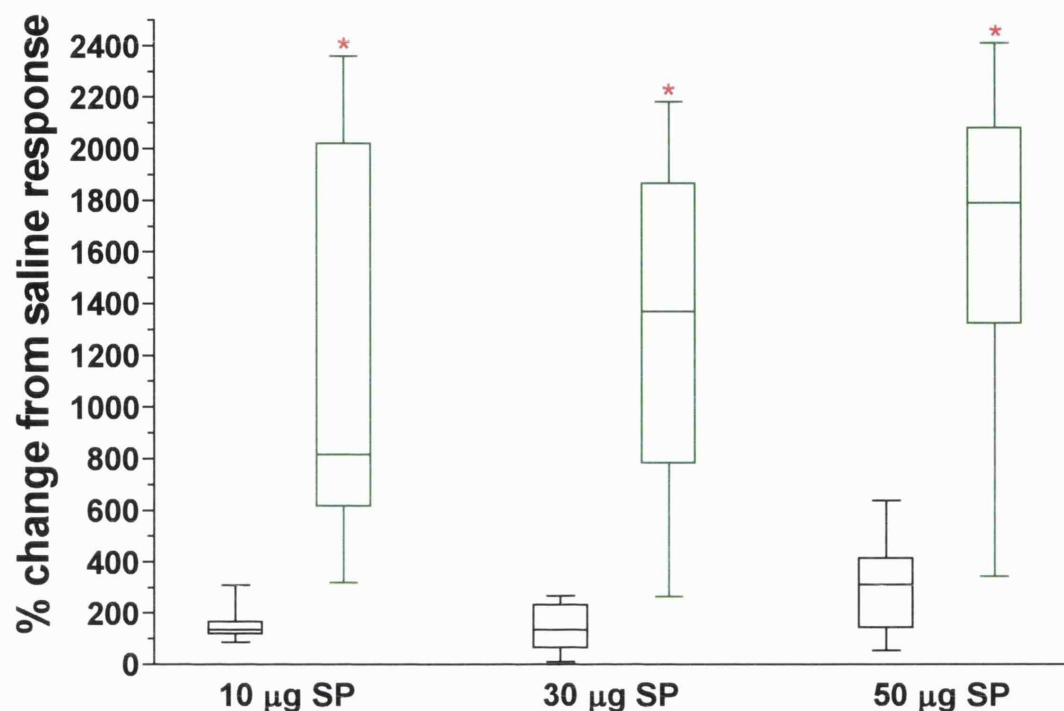


Figure 6.10: Albumin concentrations of both groups (black box for normal, $n=7$ and green for atopic, $n=7$) after substance P provocation are presented here. The data have been normalised and are expressed as percentage change from the saline response. The normalised data are presented as box and whiskers plots to compare the effect of substance P in both groups. A statistically significant difference between the two groups is indicated by * (* p -value < 0.05).

6.4 Discussion

Substance P is one of the neuropeptides that are released from sensory afferent neurones during inflammatory reactions to injury. Many inflammatory mediators such as histamine and bradykinin can also stimulate the release of substance P (Lundberg *et al.*, 1995; White *et al.*, 1989; Baumgarten *et al.*, 1997). Substance P has been shown to produce effects on blood vessels, inducing vasodilatation and vascular permeability (Laitinen *et al.*, 1987). The ability of substance P to induce histamine release from skin mast cells, and cause a wheal and flare when injected intradermally (Foreman *et al.*, 1983, 1987a and 1987c), together with its ability to cause dose-dependent bronchoconstriction in both asthmatic and normal subjects (Joos *et al.*, 1987; Crimi *et al.*, 1988), make substance P one of the most important neuropeptides

in allergic inflammation. Substance P levels in nasal mucosa have been shown to increase following antigen challenge (Mosimann *et al.*, 1993). Using autoradiography, substance P binding sites were found to be widely distributed on epithelium, submucosal glands and blood vessels (Baraniuk *et al.*, 1991c). These data, together with the diverse properties of substance P, make it a candidate likely to participate in allergic rhinitis.

Substance P has been shown, in this study, to have a predominant effect on vascular permeability since albumin was increased after all three doses of substance P, in both normal and atopic subjects. Nasal provocation with substance P induced a facial flush and an increase in superficial capillary blood flow as measured by laser Doppler flowmetry: thus substance P induced vasodilatation. The vasoactive properties of substance P in nasal mucosa have also been confirmed in other studies, and substance P induced an increase in nasal airway resistance (Devillier *et al.*, 1988; Braunstein *et al.*, 1991; Chatelain *et al.*, 1995; Fajac *et al.*, 1995; Konnon *et al.*, 1996). In nasal mucosa, therefore, substance P provocation induces vasodilatation and an increase in vascular permeability, possibly through activation of its receptors located on the vessels (Baraniuk *et al.*, 1991c).

Substance P binding sites are also present on submucosal glands (Baraniuk *et al.*, 1991c). *In vitro*, substance P induced mucous glycoprotein release from human nasal mucosal fragments (Baraniuk *et al.*, 1991c; Mullol *et al.*, 1992c). However, the present study demonstrates that substance P does not induce an increase in nasal secretion in normal subjects and in atopic subjects. No glandular secretion was observed in either group after substance P provocation. No other report, to date, has shown substance P-induced mucous or serous cell secretion *in vivo*.

In this study, substance P induced greater vascular leakage in atopic subjects than in normal subjects. Greater responsiveness to substance P in atopic subjects has also been shown for nasal airway resistance. Substance P-induced nasal airway resistance is of greater magnitude in allergic rhinitics than in the control group (Devillier *et al.*, 1988). The distribution of substance P nerve fibres is similar between rhinitic and

normal subjects; however, allergic rhinitics have a significantly higher tissue concentration of substance P (Fang *et al.*, 1998a). The increase in density of substance P in nasal mucosa and the difference in nasal response to substance P between normal and rhinitis subjects, indicate that substance P may play an important role in pathogenesis of allergic rhinitis.

A close interaction between sensory nerves and mast cells has been highlighted in neurogenic inflammation. Such an interaction has been demonstrated in human skin where antihistamines inhibited substance P-induced wheal and flare (Hagermark *et al.*, 1978; Fuller *et al.*, 1987b). Substance P has been shown to induce histamine release from human skin mast cells (Foreman *et al.*, 1987a and 1988), and *in vitro*, human nasal mast cells release histamine upon stimulation with substance P (Schierhorn *et al.*, 1995). However, *in vivo*, a number of studies were unable to demonstrate histamine release from human nasal mucosa after substance P provocation (Braunstein *et al.*, 1994; Fajac *et al.*, 1995). Treatment with the H₁ receptor antagonist, cetirizine, does not modify the nasal response to substance P, indicating that the effects of substance P in nasal mucosa are not secondary to histamine release from nasal mast cells (Braunstein *et al.*, 1994). However, substance P has been shown to enhance antigen-evoked mediator release (kinins, TAME esterase activity and histamine) from nasal mucosa (Baumgarten *et al.*, 1996). Substance P had also been shown to induce more vascular leakage in allergic rhinitics during the hay fever season than those out of the hay fever season (Braunstein *et al.*, 1991). These data further indicate the role of substance P in rhinitis, and show that during antigen exposure there is a close relationship between substance P and mast cell interaction.

The action of substance P can be limited by neutral endopeptidase (NEP). Pretreatment of nasal mucosa with the NEP inhibitor, phosphoramidon, potentiates substance P-induced facial cutaneous blood flow and nasal airway resistance (Chatelain *et al.*, 1995). In nasal mucosa, NEP is present in the epithelium, submucosal glands and blood vessels (Baranuik *et al.*, 1993; Ohkubo *et al.*, 1994), and is also found in nasal secretions (Ohkubo *et al.*, 1993). The degradation of

substance P by NEP in nasal secretion may account for the limitation of the exogenous substance P effect *in vivo*. The action of NEP may also account for the failure in detecting an increase in substance P levels after antigen challenge in other studies (Tønnesen *et al.*, 1988; Chatelain *et al.*, 1995). The positive results were obtained when protease inhibitors were added into the recovered nasal lavage fluid (Mosimann *et al.*, 1993). A decrease in NEP activity may contribute to an increase in responsiveness to exogenous substance P in allergic rhinitics out of the hay fever season or during the season.

In conclusion, substance P provocation readily induced a vascular response, but not a glandular response in the human nose. The failure of substance P to induce glandular secretion may be due to the short life of substance P, however, this seems unlikely given the other effects manifested. Systemic effects limited the dose that could be given to human nasal mucosa and precluded the use of NEP inhibitors in human subjects. From this study, therefore, substance P may have minor involvement in the secretory aspect of rhinitis.

CHAPTER 7

GENERAL DISCUSSION

Allergic rhinitis is an allergic inflammation of the nasal mucosa. It is characterized by nasal blockage, rhinorrhea, sneezing and pruritus. The aim of this study was to investigate the secretory component of the nasal response. The effects of inflammatory mediators (histamine and bradykinin) and neurotransmitters (acetylcholine and substance P) were studied and the contributions of different types of glandular secretion and plasma extravasation were analyzed.

In this study the secretory component of the nasal response was examined by quantitatively measuring the amount of secretion and its composition in nasal lavage. A simple and relatively non-traumatic method for assessing the amount of nasal secretion was developed. A cotton bud was chosen over filter paper as the material used to collect the secretion since it has a higher absorption rate and can be used to measure baseline secretion without inducing further secretion that may be caused as a result of trauma. The use of cotton buds and the protocol developed in the study enabled the time course of the effect of the challenge solution on nasal secretory activity to be followed. The use of cotton buds may also provide additional data, for example: cellular responses or the change in composition of proteins in nasal secretion. Insertion of cotton buds into the nasal cavity to collect the secretion may also be used to harvest immunological cells. The technique of obtaining cells from the adherent secretions on the cotton bud can be developed for future study. Quantitative measurement of electrolyte and protein composition can be determined by the subsequent elution of the absorbed secretion. The advantages of such techniques include not only savings of cost and time, since nasal lavage may not be necessary, but also allows the time course of the challenge solution on the cellular response or the change in protein composition in nasal secretion to be studied.

In chapter 3 methacholine was used as an indicator of the secretory response, as it is known to be a secretagogue upon submucosal glands (Mygind & Dahl, 1996; Baroody *et al.*, 1996), to evaluate the application of the technique developed using cotton buds. It was shown that the cotton bud technique is practical and capable of detecting both baseline secretion and large amounts of secretion produced by methacholine. The study in chapter 3 also compared the different mechanisms of action of methacholine, histamine and bradykinin in producing an increase in secretion from the nasal mucosa. Methacholine was shown to increase the amount of secretion mainly by inducing submucosal serous cell and mucous cell secretion. There was an increase in the plasma protein albumin, which is most likely caused by an increase in blood flow by a vasodilatation effect of methacholine, which allows plasma protein to leak via fenestrated capillaries into the nasal mucosa. Submucosal glands, a major contributor to the volume of nasal secretion, are generally controlled by the cholinergic component of the parasympathetic system. Nasal secretion induced by methacholine can be blocked by pretreatment with atropine (Druce *et al.*, 1985) and ipratropium bromide (Baroody *et al.*, 1992; Borum *et al.*, 1996; Meltzer *et al.*, 1992; Naclerio *et al.*, 1995a). Atopic patients demonstrated more responsiveness to methacholine than nonatopic subjects (Borum *et al.*, 1978; Druce *et al.*, 1985) and sensitivity to methacholine in seasonal allergic rhinitis is greater than in perennial allergic rhinitis (Prieto *et al.*, 1996). Ipratropium bromide has been widely used in patients with allergic and non-allergic perennial rhinitis with rhinorrhea as the predominant nasal symptom (Baroody *et al.*, 1992; Finn *et al.*, 1998; Kaiser *et al.*, 1998).

Histamine had been shown in chapter 3 to induce glandular secretion from both serous and mucous cells and also to increase vascular permeability. Out of the three challenge solutions studied, histamine was the only mediator that induced both sneezing and itching. In chapter 4, histamine-induced albumin release and sneeze were blocked by the H₁ receptor antagonist, cetirizine, suggesting an H₁ receptor mediated effect. The submucosal glandular responses were only partially inhibited by the H₁ receptor antagonist. The effect of histamine may be by both direct action, via histamine H₁ receptors, and indirect action (possibly by an irritant effect). Although,

the direct effect of histamine on the nasal mucosa is predominately mediated through H₁ receptor activation, the contribution of H₂ receptors in the nasal mucosa should not be overlooked. The action of histamine as a secretagogue via H₂ receptors has also been recognised, the classic effect being that of gastric-acid secretion (Black *et al.*, 1972). H₂ receptor mRNA has been shown to be localised in the nasal epithelium, serous cells and mucous cells (Hirata *et al.*, 1999). The combination of H₁ and H₂ receptor antagonists has been shown to have a greater inhibitory effect on histamine-induced increases in nasal airway resistance than using an H₁ receptor antagonist alone (Secher *et al.*, 1982). The study of the effect of an H₂ receptor antagonist on the nasal secretory response would improve our understanding of the histamine effect on the nasal secretion.

The indirect action of histamine was proposed to be through neuronal reflexes (Raphael *et al.*, 1989b; Baranuik *et al.*, 1991a; Mullol *et al.*, 1992b). The neuronal reflexes of histamine were studied in chapter 5. Histamine at doses of 300 and 1000µg induced reflex nasal secretion on the contralateral side of the nose for a brief period of time (at 6 minutes and 10 minutes). The secretion was of vascular origin. The vascular reflex responses are more sensitive than the glandular responses, since lower doses of histamine were enough to induce an increase in albumin on the contralateral side. A higher dose of histamine may be needed in order to induce a significant increase in contralateral secretion and glandular reflex responses. The vascular reflex may be due to the release of neuropeptides via an axon reflex mechanism. The effect of an H₁ receptor antagonist, local anaesthetic and atropine on the amount of contralateral secretion and protein content of the secretion could also be investigated.

Bradykinin, in contrast to methacholine and histamine, increases nasal secretion mainly by inducing an increase in vascular permeability without causing an increase in glandular secretion. Bradykinin has been shown to be more potent than histamine in inducing an increase in nasal airway resistance while the amount of secretion induced by histamine was significantly greater than that induced by bradykinin (Rajakulasingham *et al.*, 1993). The greater obstructive effect of bradykinin, compared with histamine, on nasal airway resistance may contribute to the relative lack of

efficacy of H₁ antagonists on nasal blockage. In perennial allergic rhinitis, where congestion is the primary symptom, treatment with H₁ antagonists produces only minor relief. Bradykinin has been proposed to be the mediator responsible for nasal congestion in this kind of allergy, since nasal congestion induced by antigen provocation in perennial allergic rhinitis is antagonised by Hoe-140, a B₂ receptor antagonist (Austin *et al.*, 1994c).

From the data presented in chapters 3 and 4, bradykinin produced only a vascular response via a direct action on B₂ receptors, but no glandular secretion was observed in normal subjects. The lack of a glandular response indicates that in the normal human nasal mucosa, provocation with bradykinin does not induce neuronal reflex responses that are responsible for induction of sneeze and glandular secretion, as seen with histamine. On the other hand, in allergic rhinitis subjects, nasal provocation with bradykinin also stimulates sneezing and glandular secretion via sensory-parasympathetic nerve reflexes (Baraniuk *et al.*, 1994a and 1994b; Riccio & Proud, 1996). It would be interesting to repeat the studies described in chapter 4 on bradykinin provocation, using subjects with seasonal and perennial allergies in order to show whether bradykinin could induce a glandular response in such subjects. Furthermore, bradykinin induced-glandular reflex secretion in these subjects should also be examined and the effect of Hoe 140, a bradykinin B₂ receptor antagonist, should be evaluated on this response. The effect of bradykinin on the human nasal mucosa has been proposed to involve generation of histamine, since pretreatment with histamine H₁ receptor antagonists significantly reduced the bradykinin-induced increase in nasal airway resistance (Austin *et al.*, 1996). The contribution of histamine to the action of bradykinin on the secretory component in the human nasal mucosa of normal and allergic rhinitis subjects, and to the bradykinin-induced glandular reflexes in allergic rhinitis could be examined. The induction of sneezing and glandular secretion in allergic rhinitis following bradykinin provocation demonstrated that in allergic inflammation there is an enhancement in neuronal responsiveness, which may cause nasal hyperresponsiveness to bradykinin.

The study of substance P described in this thesis, indicated that substance P may make only a minor contribution to the induction of nasal response in both normal and allergic rhinitis. However, this does not exclude the possibility that substance P may be associated with the pathology of allergic rhinitis. Substance P has been shown to enhance antigen-evoked mediator release, kinin, TAME esterase and histamine from the human nasal mucosa (Baumgarten *et al.*, 1996). Twenty four hours after nasal challenge with pollen in seasonal allergic rhinitis, substance P provocation induced a further increase in the number of eosinophils which were already numerous before substance P challenge (Fajac *et al.*, 1995). It is therefore possible that substance P may make some contribution to the induction of the late phase reaction in allergic rhinitis.

CHAPTER 8

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